IL-6-Engineered DC Stimulate Efficient Antitumor Immunity via Enhanced and Prolonged T Cell Cytotoxicity and Survival

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ABSTRACT

Dendritic cells (DCs) modified by some immunomodulatory genes can stimulate a strong antitumor immunity and improve the treatment of tumor cells on the condition that the sources of tumor-associated antigens (TAAs) are available. IL-6, a pleotropic cytokine, has been found to inhibit $CD4^+25^+$ regulatory T (Treg)-cell-mediated immune suppression and decrease activation-induced cell death (AICD) without interfering the process of T-cell activation. To enhance DC-based cancer vaccine, we engineered DCs to express transgene IL-6.

We constructed a fiber-modified recombinant adenovirus vector $AdV_{IL\text{-}6}$ expressing IL-6, infected DCs with $AdV_{IL\text{-}6}$, and then investigated the efficacy of antitumor immunity induced by vaccination with DCs engineered to express IL-6 transgene. We demonstrated that DCs infected with the recombinant adenovirus $AdV_{IL\text{-}6}$ induced DC maturation by up-regulation of the expression of MHC class Ⅱ (I_a^b), CD40, CD54 and CD80 expression. We also demonstrated that vaccination of OVA-pulsed $AdV_{IL\text{-}6}$-infected DCs ($DC_{OVA/AdVIL\text{-}6}$) was able to stimulate a stronger OVA-specific effector $CD8^+$ cytotoxic T lymphocyte (CTL) response than vaccination with the control virus AdV_{pLpA}-infected DCs ($DC_{OVA/AdVpLpA}$). More importantly, vaccination of mice with $DC_{OVA/AdVpLpA}$ could protect 100% mice from intravenous (i.v.) challenge of a low dose (0.5×10^5 cells per mouse, 8/8 mice protected) of OVA-expressing BL6-10_{OVA} tumor cells, but only 63% mice from i.v. challenge of a high dose (1×10^5 cells per mouse, 5/8 mice protected) of BL6-10_{OVA} tumor cells. However,

vaccination of $DC_{OVA/AdVIL-6}$ induced an augmented antitumor immunity *in vivo* by complete protection of mice (8/8) from challenge of both low and high doses of BL6-10_{OVA} tumor cells.

To study the immune mechanism underlying the result of IL-6 engineered-DC vaccine, we generated the $DC_{OVA/AdVIL-6}$-activated OTI $CD8^+$ T cells and $DC_{OVA/AdVpLpA}$-activated OTI $CD8^+$ T cells. We demonstrated that $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells displayed a higher level of CD62L, FasL and perforin than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells. $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells had a prolonged T cell survival after they were transferred into C57BL/6 mice. Furthermore, the results of the animal study showed that 100% of mice bearing OVA-expressing EG7 tumors (8mm in diameter, 8 mice per group) were tumor-free after they were i.v. treated with $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (2×10^6 cells per mouse). However, the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells failed in eradication of EG7 tumors in all 8/8 mice.

Taken together, Adenovirus-mediated IL-6 transgene engineered DC vaccine stimulates efficient $CD8^+$ T cell responses and antitumor immunity via enhanced T cell cytotoxicity and prolonged T cell survival. DCs engineered to express IL-6 by adenovirus-mediated IL-6 gene transfer may offer a new strategy in production of DC cancer vaccines.

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2-ME	2-mercaptoethanol
aa	amino acid
Ab	Antibody
ACS	American Cancer Society
AdV	Adenovirus
Ag	Antigen
AICD	Activation-induced cell death
AP-1	Activator protein 1
APC	Antigen presenting cell
ATCC	American type culture collection
ATP	Adenosine triphosphate
BAGE	B melanoma antigen
BCG	Bacillus calmette-guerin
BCR-ABL	Breakpoint cluster regional/antigen-binding lymphocyte
BFGF	Basic fibroblast growth factor
BGH	Bovine growth hormone
BM	Bone marrow
BM-DC	Bone marrow derived dendritic cell
bp	base pairs
BSA	Bovine serum albumin
CAR	Coxsackie virus and Adenovirus receptor
CD40L	Co-stimulatory molecule 40 ligand
CFSE	Carboxyfluoroscein diacetate succinimidyl ester
CMV	Cytomegalovirus
CPE	Cytopathic effects
CTL	Cytotoxic T lymphocyte
dATP	2’deoxyadenosine 5’-triphosphate
DC	Dendritic cell
DC2.4	DC cell line 2.4
ds	double stranded

DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Early transcribed regions
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetracetic acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Minimal essential medium with earle's salts
EPB	Enhancer-binding protein
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
Flt-3L	Fms-like tyrosine kinase 3-ligand
FoxP3	Fork-head/winged helix transcription factor
GAGE	G melanoma antigen
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	glycoprotein
HBV	hepatitis B viruses
HCV	hepatitis C viruses
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
HER	Human epidermal growth factor receptor
HHV-8	Human herpes virus type 8
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSP	Heat shock protein
HYLV-1	Human T-cell lymphotropic virus type I

i.t.	intratumoral
i.v.	intravenous
ICAM	Intracellular adhesion molecule
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IL	Interleukin
IL-6R	Interleukin -6 receptor
IP	Interferon-y-inducible protein
ITPG	Isopropylthio-β-D- galactoside
ITR	Inverted terminal repeat
JAK	Janus kinase
kb	kilobase pairs
Kd	Kilo-dalton
L	Late transcribed regions
LB	Lauria-bertani
LPS	Lipopolysaccharide
Lptn	Lymphotactin
LTR	Long terminal repeat
mAb	monoclonal antibody
MAGE	Melanoma antigen
MART-1	Melan-A/melanoma antigen recognized by T cell
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MHC I	MHC class I
MHC II	MHC class II
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
MUC	Mucin
Mut	Mutator

NEB	New England biolabs
NF	Nuclear factor
NK	Natural killer
ori	origin of replication
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
PFU	Plaque forming unit
PI3K	Phosphatidylinositol-3-kinase
pMHC	peptide-MHC complex (SIINFEKL-MHC class I complex)
Rb	Retinoblastoma
RGD	arginine-guanine-aspartate
RT-PCR	Reverse transcriptase-polymerase chain reaction
s.c.	subcutaneous
SDS	Sodium dodecyl sulfate
SLC	Secondary lymphoid tissue chemokine
SSX	Synovial Sarcoma on X chromosome
STAT	Signal transducer and activator of transcription
TAA	Tumor associated antigen
TAE	Tris-acetate EDTA
TB	Terrific broth
TCR	T cell receptor
TE	Tris EDTA
TGF	Transforming growth factor
Th	T helper
Th1	T helper type I
Th2	T helper type II

TLR	Toll-like receptor
Tm	memory T cells
TMB	3,3’,5,5’-tetramethylbenzidine
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell
TRP	Tyrosinase-related protein
VAP-1	Vascular adhesion protein 1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
β-ME	β-mercaptoethanol

Chapter 1

REVIEW OF THE LITERATURE

1.1 Introduction

Cancer is a severe disease and has a very high risk of death. The World Health Organization (WHO) reported that cancer was responsible for about 13% of all deaths in 2007. Based on data from the American Cancer Society (ACS), 7.6 million people died from cancer worldwide in 2007. We have spent a lot of money and time to find out effective treatments to conquer this disease. However, still there are many problems with cancer therapy that need to be addressed. Today, the most frequently used treatments for malignant tumors consist of surgery, chemotherapy and radiation therapy. Theoretically, surgery is able to cure non-hematological cancer by complete removal of tumor cells. However, once tumor cells have metastasized to distant sites, surgical therapy is usually not viable. Both chemotherapy and radiation lack specificity for tumor cells, and cause damage to normal tissue, resulting in severe side-effects. The goal of cancer treatment is to remove cancer cells completely without damaging normal cells. Thus, a specific treatment approach is required for curing cancer. In past decades, research has focused on immune cell-based strategies for the treatment of human cancer. Cancer immunotherapy is able to eliminate the tumors via immune system responses. This therapy may reach the goal of cancer treatment without damage to normal tissues.

1.2 Immune Responses

The immune system in animals and humans is a collection of mechanisms to prevent the infection of pathogens, such as bacteria, viruses and tumor cells. The innate immune system and the adaptive immune system are the two components of the immune system. The innate immune system, found in nearly all forms of life, responds in a non-specific manner. In the innate immune system, infectious agents are recognized and killed by phagocytic cells. Some phagocytic cells, such as natural killer (NK) cells, dendritic cells (DCs) and macrophages, are not only involved in the innate immune system, but also participate in the adaptive response. These cells form a bridge between these two kinds of immune systems. Unlike innate immune responses, the adaptive immune system, only found in jawed vertebrates, can lead to antigen-specific responses and immunological memory. In the adaptive immune responses, powerful antigen presenting cells (APCs), such as DCs, macrophages and B cells, are required to recognize the specific "non-self" antigens and then generate immune responses. Two kinds of immune responses are included in the adaptive immune system, cellular immunity and humoral immunity. Humoral immunity refers to the production of secreted antibodies, which are produced by activated B cells. The process of B cell activation involves helper T cell interaction with B cells as well as cytokine productions. These antibodies can eliminate their specific antigen via pathogen and toxin neutralization, classical complement activation, opsonization and phagocytosis. In cellular immunity, T lymphocytes are activated via APCs. These activated T lymphocytes kill phagocytosed microbes and lyse infected cells. After the initial immune response, B and T memory cells appear in the blood and will

bring a stronger and more effective immune response upon repeat encounters with the same pathogen.

1.3 Tumor-associated Antigens (TAAs)

Tumor-associated antigens (TAAs), which can be targeted using T cell-immunotherapy, play an important role in the adaptive immune system. These antigens, produced in some tumor cells and also some normal cells, trigger an antitumor immune response in the host. Serological expression cloning is a technology for identification of TAAs from different tumors. Their ability to identify tumor cells makes TAAs potential candidates in cancer therapy. TAA contain several categories (1):

Shared tumor-specific antigens are a group of tumor antigens and include melanoma antigen (MAGE)-type gene families (MAGE-1 to 10, B melanoma antigen (BAGE), G melanoma antigen (GAGE) and synovial sarcoma on X chromosome (SSX)-1 to 9) (2). They are usually silent in normal tissues except in testis, but are expressed in a broad range of tumors, particularly melanomas and carcinomas of the lung, head, neck, bladder, and esophagus. However, their expression is absent or infrequent in renal carcinoma and leukemia.

Some melanoma-reactive T cells can recognize melanocyte differentiation gene products, including melan-A/melanoma antigen recognized by T cell (MART-1), glycoprotein-100 (gp100), tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2. These antigens can be targeted if they are significantly over-expressed in malignant cells or if the normal cells involved are not functionally important. These antigens are also

present in normal melanocytes. T cell recognizes melanocyte differentiation antigens in a conventional class I and class II restricted manner. These antigens are the frequently used target of melanoma-reactive T cells. However, the activated T cell will cause autoimmunity in the form of vitiligo, or less frequently uveitis (3, 4).

Viruses are recognized as a major etiologic factor in human cancer. The percentage of virus-induced cancer is 15% to 20% (5, 6). Viruses involved in human cancers are Epstein-Barr virus (EBV), human T-cell lymphotropic virus type I (HYLV-1), human herpes virus type 8 (HHV-8), hepatitis B and C viruses (HBV and HCV, respectively), and human papillomavirus (HPV) type 16 and 18 (and other less prevalent oncogenic-HPV types). Some antigens from these oncogenic viruses can be targeted in the immune system.

Some oncoprotein /receptors and mutations, which occur in oncogenes and tumor suppressor genes, meet the two important criteria of a good tumor antigen :(i) over-expression in the tumor and low expression in normal cells or unique expression in the tumor cell that allows the immune system to distinguish cancer cells from normal cells and (ii) mandatory function of the protein for malignant transformation, preventing the tumor from escaping the immune response by suppressing expression of the antigen without losing its malignant phenotype (1). For these reasons, antigens from this group, such as human epidermal growth factor receptor (HER)-2/neu, mutant ras or mutant p53, are probably the most ideal and have been widely studied as potential tumor antigens against which immunotherapeutic vaccines are targeted. Mutant ras are present in a broad spectrum of cancers, including adenocarcinoma of the pancreas, colorectal

adenocarcinoma, lung adenocarcinoma, thyroid tumor and myeloid leukemia, but are not present in normal cells (7). HER-2/neu is over-expressed in approximately 25-30% of invasive breast cancers (8, 9) and in ovarian (10) and lung cancers (11).

Finally, carbohydrate antigens, which are expressed as glycolipids or glycoproteins at the tumor cell surface, have proven to be potent targets for antibody recognition. These antigens also attack cancer cells due to their unexpected immunogenicity and their abundance at the normal cell surface. GM2, GD2 and GD3 are three of the most well known melanoma glycoproteins. These three glycoproteins are also expressed in sarcoma neuroblastoma, colon, ovary and stomach cancer.

1.4 Dendrite cells (DCs)

DCs, which are powerful APCs, uptake antigens efficiently via macropinocytosis, receptor-mediated endocytosis and engulfment of apoptotic bodies. There are four stages of development in the life cycle of DCs, including DC progenitors, precursor DCs, immature DCs and mature DCs. The DC progenitors in the bone marrow can develop to be circulating precursor DCs. After encountering an antigen (Ag), these DCs induce secretion of cytokines, such as interferon- α (IFN-α), to activate the eosinophils, macrophages and NK cells for the innate immune response and also enter tissues to become immature DCs for induction of the adaptive immune response. Tissue-residing immature DCs can capture Ag due to their high endocytic and phagocytic capacities. In the periphery, immature DCs capture antigens and migrate to secondary lymphoid organs. In these locations, immature DCs develop to mature DCs, expressing high levels of major

histocompatibility complex (MHC) class II molecules, costimulatory molecules, such as CD80, CD86, CD40, and adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, as well as some cytokines. Mature DCs select rare circulating antigen-specific lymphocytes and activate these immune effector cells, including the $CD8^+$ cytotoxic T cells, $CD4^+$ T helper (Th) cells and B cells. Mature DCs have high stimulatory activity for T lymphocyte cells. In the mixed lymphocyte reaction, one DC could activate three thousand naive T cells. After activation, T lymphocytes migrate to the injured tissue where the cytotoxic T cells will lyse the infected cells and the Th cells will activate macrophages, NK cells, and eosinophils (12) through secreting some cytokines. The activation of B cells requires the interaction of the activated Th cells and mature DCs. After activation, B cells mature into plasma cells and migrate into different areas to produce antibodies. Finally, mature DCs die via apoptosis after interaction with lymphocytes (13).

Based on the above description, DCs also play an important role in both the innate and adaptive immunity of the antitumor immune response. In innate immunity, after recognizing tumor pathogen associated molecular patterns (PAMP), the circulating precursor DCs release IFN-α to activate macrophages, NK T cells, and NK cells. These cells kill tumors, leading to the release of tumor cell bodies. By capturing the TAAs released by those tumor cell bodies, the tissue-residing immature DCs become mature and display tumor antigens for selection of tumor specific T lymphocytes, including $CD4^+$ and $CD8^+$ T cells. The activated TAA-specific T cells home to the tumor sites and eliminate these tumor cells, inducing a cellular immune response(13). Based on these

powerful important roles of DCs, DC-based vaccines have been proposed as a treatment modality for cancer.

1.5 Dendrite cell (DC) –based Vaccines

1.5.1 Tumor Antigen-pulsed DC Vaccines

It has been suggested that the tumor-antigen-pulsed DCs can induce the development of MHC- class I (MHC I) - and class II (MHC II)-specific T cell responses *in vitro* and *in vivo*. Tumor antigen-pulsed DC vaccine development includes two kinds of approaches, pulsing DCs with specific tumor peptide and loading DCs with total tumor lysate antigens.

It is reported that when delivered to animals, vaccines consisting of DCs pulsed *in vitro* with peptide antigen can induce antigen-specific, cytotoxic T lymphocyte (CTL) - mediated protection against lethal tumor challenges. Moreover, these DCs pulsed *in vitro* with peptide antigen elicit a therapeutic antitumor immunity *in vivo* (14). The tumor specific antigen-pulsed DCs have been used in animal studies and clinical trials, and have been shown to have more or less beneficial effects. The MHC-restricted synthetic tumor specific peptides that were used in animal models include Mutator (Mut) I (15), Ovalbumin (OVA) (15), gp70/p15E (16) HER2/neu (17) and fusion protein breakpoint cluster regional/antigen-binding lymphocyte (BCR-ABL) (18). In clinical trials, DCs pulsed with melanoma related antigens have been used to treat melanoma tumors and this vaccination has induced antigen-specific immunity (19-21) Furthermore, the HER/neu (22) and MUC-1 (23) were used to pulse DCs to cure tumors that over-express these two

antigens. In patients with these tumors, the effector immune responses can be induced by these vaccinations.

However, vaccination with DCs pulsed by specific tumor peptides only stimulate a CTL response in the immune system and have no effect on MHC class-Ⅱ-restricted Th cells which initiate and sustain immune responses. Additionally, it is difficult to identify and generate a specific antigen peptide for some tumors. Based on these two points, whole tumor lysate antigens were used to pulse DCs *in vitro*. The use of whole tumor lysate antigens as a source of antigen to pulse DCs induces not only CTL responses, but also helper T cell responses, which is important to achieve a more complete anti-tumor response. Therefore, DCs loaded with whole tumor lysate antigens are very effective for tumor vaccine strategies. It was reported that in two separate strains of mice with histological distinct tumors, DCs pulsed with whole tumor lysate antigens mediate potent antitumor immune responses *in vitro* and *in vivo* (24). In clinical trials, vaccinations using DCs loaded with whole tumor lysate antigens have been shown to be valuable in the treatment of malignant melanoma (19), brain tumor (25, 26) uterine serous papillary cancer (27), renal cell carcinoma (28, 29)and pediatric solid tumor (30).

1.5.2 Tumor mRNA-pulsed or Transfected DC Vaccines

DCs pulsed or transfected with tumor mRNA translate the respective tumor proteins and induce CTL responses. The advantages of these vaccines are that: (i) these vaccines can work irrespective of the patient's human leukocyte antigen (HLA) repertoire because these translated tumor proteins have wide HLA specificities and (ii)It is easy to isolate

the tumor mRNA from animal or human tumor cells and amplify these mRNAs conveniently without loss of function (31, 32). There are two approaches to deliver the tumor mRNA to DCs, including mRNA lipofection and mRNA electroporation into human hematopoietic cells. Although the latter method possesses a higher efficiency, mRNA/liposome complexes are more widely used (33, 34). Tumor mRNA-pulsed or transfected DCs can encode not only the tumor antigen but also the costimulatory molecular. During this process, the DCs become mature and mediate the tumor antigen peptide to MHC I and MHC II molecules. Finally, T cell responses are induced by this DC vaccine.

It was reported that murine DCs transfected with RNA amplified from the melanoma B16/F10.9 cell line can induce CTL responses in mice and stimulate protective immunity in tumor-bearing mice (31). Shigeo Koido *et al* reported that vaccination of wild-type mice with MUC1 RNA-transfected DCs induced anti-MUC1 immune responses against MUC1-positive, but not MUC1-negative, MC38/MUC1 tumor cells. Mice immunized with the transfected DCs were protected against challenge with MC38/MUC1 tumor cells. Furthermore, the established MC38/MUC1 tumors in mice were eliminated after receiving the vaccination (35). During clinical trials, there are some limitations with tumor mRNA-pulsed or transfected DC vaccines, which have been used in the treatment of colorectal cancer (36), bladder cancer (37) and renal tumors (38). However, Axel Heiser *et al* found that total tumor RNA-transfected DCs may represent a widely applicable vaccine strategy to induce potentially therapeutic polyclonal T-cell responses in cancer patients (38).

1.5.3 Necrotic or Apoptotic Tumor Cell-loaded DC Vaccines

DCs can recognize and uptake apoptotic and necrotic tumor cells via specific receptors, such as αvβ5, CD36 or the phosphatidylserine receptor for apoptotic tumor cells (39), and CD91, the receptor for heat shock protein (HSP) expressed on necrotic tumor cells (40, 41). After uptake of the apoptotic and necrotic tumor cells, DCs present or cross-present both MHC I and MHC II epitopes of a defined tumor antigen or multiple tumor antigens (42-44). This vaccine does not rely on the HLA haplotype and can be utilized for all patients. The stage of apoptosis of the tumor cell significantly affects DC maturation and induction of antitumor immunity. It has been shown that only late-phase, not early-phase, apoptotic tumor cells can stimulate DC maturation and lead to the induction of antitumor immunity (45). Moreover, because DCs recognize and uptake necrotic tumor cells via CD91 and HSP, the level of HSP exposed on necrotic tumor cells plays a key role in the maturation of DCs and stimulation of antitumor immunity (46, 47). Our lab reported that DC-mediated phagocytosis of necrotic or apoptotic tumor cells induces DC maturation, resulting in the up-regulation of pro-inflammatory cytokines (interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF- α), IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and chemokines (macrophage inflammatory protein (MIP)-1α, MIP-1β and MIP-2), and the expression of co-immunostimulatory molecules (CD40 and CD86). Moreover, our data also showed that vaccination with DCs that had phagocytosed apoptotic/necrotic BL6-10 cells was able to more powerfully stimulate allogeneic T-cell proliferation *in vitro* than vaccination with DCs pulsed with tumor peptide, and induce strong protective immunity against BL6-10 tumor challenge in

animal models (48). The vaccinations of necrotic or apoptotic tumor cell-loaded DCs had been studied in melanoma cells (42), leukemia cells (49) and squamous cell carcinoma (50) in animal models. All of these vaccines can significantly suppress tumor growth and appear to offer new strategies in DC cancer vaccines.

1.5.4 Transgenic DC vaccines

In animal models, vaccination with DCs pulsed with tumor peptides, lysates, mRNA or loaded with apoptotic/ necrotic tumor cells induce significant antitumor CTL responses and antitumor immunity. However, the results from early clinical trials pointed to a need for an additional improvement of DC-based vaccines because of the difficulty in preparation of such materials from human solid tumors. The transgenic DC vaccine is a new strategy that can overcome these technical difficulties. The target genes transferred into the DCs fall into two categories, TAAs and immunomodulatory proteins, such as cytokines or costimulatory molecules. Among the many methods that have been used to introduce genes into DCs, the adenovirus (AdV) vector was known to be a good candidate due to high infection efficiency and the low risk associated with mutagenesis insertions.

1.5.4.1 DCs Engineered to Express Tumor-associated Antigens

DCs engineered to express tumor-associated antigens have several advantages over DCs pulsed with tumor antigen proteins and peptides. First, DCs engineered to express TAAs are more sensitive to the immunologic relevance of individual cancer-specific peptides as long as the molecules transduced into the DCs are immunogenic. Second, the tumor

proteins that are synthesized within the DCs will permit specific antigen presentation to T cells for longer periods without the breakdown of the peptide/MHC complex (pMHC). It has been reported that DCs engineered to express TAAs have a more potent ability to prime the antitumor immune response in animal models than the DCs pulsed with tumor antigen proteins both *in vitro* and *in vivo* (51). Third, DCs engineered to express TAAs can generate $CD8^+$ T cell responses against multiple class-I restricted epitopes within the antigens, resulting in a wide-ranging antitumor response (52). It has been demonstrated that vaccination using engineered DCs enhanced antitumor immunity via activation of $CD8^+$ T cells (53). MART-1 (54), gp100 (55), p53 (56) and MUC-1 (51) have been used to transfect murine and/or human DCs to induce tumor antigen-specific immune responses.

HER-2/neu (HER2 or c-erb-B2) antigen represents an ideal therapeutic target for breast cancer. It is commonly amplified and/or overexpressed in breast cancer, ovarian cancer and lung caner in humans. Y Chen *et al* have reported that genetic immunization using DCs transduced *ex vivo* with an adenovirus expressing the ErbB-2/neu gene (AdNeuTK) can induce protective and therapeutic immunity against a breast tumor cell line over-expressing ErbB-2/neu (57). Moreover, vaccination with DC/AdNeuTK can cure mice with established tumors. This therapeutic effect is enhanced by co-transduction of the DCs with AdV vector encoding murine IL-12 (AdmIL-12) (57). Our lab has reported that the use of fiber-modified AdVneu-transduced DCs increased neu expression compared to non-modified AdVneu-transduced DCs. Fiber-modified DCneu showed up-regulation of MHC II, co-stimulatory, adhesion molecules as well as pro-inflammatory cytokines,

stimulated a higher percentage of HER-2/neu-specific $CD8^+$ T cells, a stronger neu-specific CTL response, and a much stronger T helper type I (Th1)/ T helper type II (Th2) mixed response than non-modified DCneu. Moreover, in parental FVB/N and FVB/neuN Tg mice, vaccination with fiber-modified DCneu induced more efficient protective immunity than DCneu (58). In the treatment of breast cancers, the fiber-modified DC vaccine induced stronger anti-HER-2/neu immune response, because the fiber modification increases *ex vivo* trsnsfection efficiency.

1.5.4.2 DCs engineered to express immunomodulatory molecules

As mentioned above, DCs engineered to express TAAs are more effective than those simply pulsed with tumor peptides. However, it is difficult to choose appropriate tumor antigens as DC transduction candidates. To augment the ability of DCs to stimulate a strong antitumor immunity, some immunomodulatory proteins such as cytokines and chemokines have been transducted into DCs. Engineered DCs expressing a T cell stimulatory cytokine can improve the treatment of tumor cells on the condition that the sources of TAAs are available. Additionally, compared to modified tumor cells, modified DCs can provide more potent vaccines because the DCs are professional APCs and can stimulate T cell antitumor activity. It has been demonstrated that immunomodulatory gene-modified DCs expressing some immune stimulatory cytokines or chemokines have been used as DC-based vaccines for cancer in animal models and induce stronger antitumor activity.

GM-CSF is an essential growth and differentiation factor for DCs in culture. It has been reported that administration of GM-CSF *in vivo* augments primary immune responses (59). Therefore, GM-CSF may be an ideal candidate to enhance the effectiveness of DC-based vaccines. Clara Curiel-Lewandrowski *et al* reported that GM-CSF-transfected bone marrow derived dendritic cells (BM-DCs) displayed high level expression of MHC and costimulatory molecules. These engineered DC showed a high alloantigen or peptide antigen-presenting capacity *in vitro*. Moreover, the result of *in vivo* immunizations showed GM-CSF-transfected BM-DCs increase their Ag-presenting capacity and antitumor immunity, which was correlated with a better migratory capacity (60).

IL-12 is naturally secreted by DCs (61), macrophages and human B-lymphoblastoid cells in response to antigenic stimulation. It enhances NK cell and CTL activities, plays a key role in the induction of Th1-type immune responses including IFN-γ production (62) and has IFN-γ/IFN-inducible protein 10-dependent anti-angiogenic effects (63, 64). Intratumoral (i.t.) injection with retrovirus IL-12 gene-modified BM-DCs could significantly suppress the growth of established tumors and induce a strong antitumor T-cell response (65).

IL-7 is a hematopoietic growth factor produced by the stromal cells of the red marrow and thymus. It plays an important role in proliferation during certain stages of B-cell maturation, T and NK cell survival, development and homeostasis. It has been reported that i.t. injection of adenoviral IL-7-transduced dendritic cells ($DC_{AdVIL\text{-}7}$) resulted in complete tumor regression in two murine lung cancer models. Moreover, after complete

tumor eradication, those mice treated with DC-AdV_{IL-7} showed significantly greater release of splenocyte GM-CSF and IFN-γ (66).

In our lab, we have also researched the possibility of inducing antitumor immunity using engineered DCs that can express Fms-like tyrosine kinase 3 ligand (Flt3L) (67), TNF-α (68) or co-stimulatory molecule 40 ligand (CD40L) (69).

Flt3L is a regulator of hematopoietic cell development. It can drive DC development along both the lymphoid and myeloid developmental pathways from $Flt3^+$ progenitors to $Flt3^+$ DCs (70). Flt3L plays an important role in the expansion of both DCs (71) and NK cells (72, 73). Our data showed that AdV_{Flt3L} infection enhances cytokine (IL-1β) and chemokine (MIP-1α, MIP-1β, Interferon-y-inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1 and MIP-2) expression, but not maturation of DCs. AdV_{Flt3L} infection also stimulated adenovirus Flt3L gene-modified BM-DC (DC_{Flt3L}) proliferation *in vitro* and induced their migration toward regional lymph nodes *in vivo*. It has also been demonstrated that DC_{Flt3L} cells were able to stimulate a type 1 immune response comprising $CD4^+$ Th1 and $CD8^+$ Tc1 activation and induce the enhanced tumor-specific cytotoxic T lymphocyte (CTL) and non-specific NK responses, compared to control virus-infected DCs (DC_{pLpA}) and un-infected DCs. Vaccinations with Mut1-pulsed DCs, DC_{pLpA} and DC_{Flt3L} protected mice from a low dose (0.5×10^5) challenge of 3LL tumor cells. However, only vaccination with the Mut1-pulsed DC_{Flt3L} was able to protect 63% (6/8) mice from a high dose (3×10^5) challenge of 3LL tumor cells. Therefore, vaccination with Mut1-pulsed DC_{Flt3L} cells induced enhanced antitumor immunity *in vivo*, even against a high dose of 3LL tumor cells (67).

TNF-α is a multifunctional, immunoregulatory cytokine with a broad spectrum of activities, including induction of $CD8^+$ T cell proliferation (74) and cytotoxicity (75), and provision of costimulatory survival signals for $CD8^+$ T cells (76). In addition, it also can stimulate DC maturation (77), reduce/counteract IL-10-mediated DC inhibition (78), and inhibit the suppressive function of $CD4^+CD25^+$ regulatory T (Treg) cells by down-regulation of fork-head/winged helix transcription factor (FoxP3) expression (79). Our data show that DCs infected with recombinant adenovirus $AdV_{TNF\alpha}$ ($DC_{TNF\alpha}$) displayed a greater maturation than control DCs cultured in exogenous TNF-α. $DC_{TNF\alpha}$ up-regulated expression of pro-inflammatory cytokines (e.g. IL-1β and IL-18), chemokines (e.g. INF-γ-inducible protein-10 and MIP-1β), the CC chemokine receptor CCR7, and immunologically important cell surface molecules (CD40, CD86 and ICAM-1). These transgenic DCs stimulated stronger allogenic T-cell responses *in vitro* and T-cell activation *in vivo*, displayed enhanced chemotactic responses to the MIP-3β *in vitro*, and trafficked into the draining lymph nodes more efficiently than the control DCs. Vaccination of mice with Mut1 peptide-pulsed $DC_{TNF\alpha}$ induced more efficient *in vitro* Mut1-specific $CD8^+$ cytotoxic T-cell responses and stimulated stronger anti-solid tumor immunity *in vivo* than control DCs cultured in exogenous TNF-α (68). Moreover, our data also show that vaccination with $DC_{TNF\text{-}\alpha}$ cells pulsed with the OVA I peptide can reduce growth of the small (3–4 mm in diameter), but not large (6–8 mm in diameter), established MO4 (melanoma cell line) tumors. However, the large MO4 tumors in 3 out of 8 (38%) mice were eliminated through treatment with a combination of $AdV_{TNF\text{-}\alpha}$-mediated gene therapy and TNF-α-gene-engineered $DC_{TNF\text{-}\alpha}$ vaccination. This indicates

that the combined immunotherapy strategy is much more efficient in curing well established tumors than mono-therapy with a DC vaccine alone (80).

CD40L, a 33-kDa type II membrane protein, is a member of the tumor necrosis factor (TNF) gene family that is expressed on activated $CD4^+$ T cells (81, 82). The receptor for CD40L, CD40, is expressed on DCs. The CD40–CD40L interaction has been reported to be essential for activation of DCs via stimulation by $CD4^+$ Th cells, with up-regulated expression of costimulatory CD80 molecule and ICAM-1 and secretion of IL-12, which can trigger $CD8^+$ CTL responses (83, 84). Our data show that infection of DCs with recombinant adenovirus AdV_{CD40L} resulted in activation of DCs with up-regulated expression of proinflammatory cytokines (IL-1β and IL -12), chemokines (IP -10, and MIP-1α), and immunologically important cell surface molecules (CD54, CD80, and CD86). Our data also demonstrate that DCs infected with AdV_{CD40L} (DC_{CD40L}) are able to stimulate enhanced allogeneic T- cell proliferation and Mut1- specific $CD8^+$ CTL responses *in vitro*. Mut1 peptide-pulsed control virus–infected DC (DC_{pLpA}), could only protect mice from a low dose challenge of 3LL tumor cells. However, vaccination with Mut1 peptide-pulsed AdV_{CD40L}–infected DC_{CD40L} induced a stronger antitumor immunity *in vivo* and protected mouse completely from challenge of both low and high doses of 3LL tumor cells (69).

Chemokines are a family of small cytokines secreted by some cells. The major role of chemokines is to act as a chemo-attractants to guide the migration of cells. In the immune system, chemokines may enhance the ability of DCs interacting with T cells. Chemokines play an integral role in the initiation of specific immune responses (85). Since DCs

express some chemokine receptors (86, 87), chemokines participate in the migration and recruitment of DCs (88, 89). Therefore, the genetic modification of DCs with chemokines can increase their antitumor immunity.

Lymphotactin (Lptn), a C chemokine, specifically attracts both NK cells and T cells *in vivo* (90, 91). Adenoviral Lptn -transduced dendritic cells (DC_{Lptn}) have been shown to induce a strong protective and therapeutic antitumor immunity. Immunization with a low dose (1×10^4) of Mut1 peptide-pulsed DCs induced a weak CTL activity, whereas the same amount of Mut1 peptide-pulsed DC_{Lptn} greatly increased specific CTL against 3LL tumor cells. Vaccination with 1×10^4 peptide-pulsed DC_{Lptn} could completely protect mice from 5×10^5 3LL tumor cell challenge, but the peptide-pulsed DC could not. Moreover, the pulmonary metastases of mice bearing 3LL tumor were significantly reduced with treatment of 1×10^4 Mut1 peptide-pulsed DC_{Lptn}, whereas the same low dose of Mut1 peptide-pulsed DCs had no obvious therapeutic effects (92).

Secondary lymphoid tissue chemokine (SLC), a CC chemokine, is able to recruit both DCs and naïve T cells via the CCR7 receptor found on both cell types (93, 94). Based on this, SLC is believed to play an important role in the initiation of an immune response by promoting the interaction of naïve T cells and the antigen that DCs present in the lymph node. Christopher J. Kirk *et al* reported that i.t. injection of SLC-expressing DCs (DC_{SLC}) more strongly inhibits tumor growth than either control DCs or SLC alone. Distant site immunization of tumor bearing mice with tumor lysate pulsed DC_{SLC} can stimulate an antitumor response whereas control DCs could not. They also found that subcutaneous (s.c). injection of lysate-pulsed DC_{SLC} promoted the migration of T cells to the

immunization site. This report demonstrates that SLC expression by DCs can induce antitumor responses and enhance antitumor immunity (95).

1.5.5 Problems in DC-based Vaccine

In recent years, many key confounding issues with DC-based vaccines have been identified, which will ultimately lead to improving the feasibility and efficacy of the approach in years to come. These key problems arise mainly because of the specific characteristics of solid tumors that can evade both innate and adaptive immunity, such as: (i) tumor-induced T cell apoptosis by expression of FasL (96); (ii) prevention of T cells infiltration by tumor neovasculatures (97); (iii) lack of Th responses (98); and (iv) immune suppression related to tumoral IL-10 expression (99, 100)(28, 29) and recruitment of Treg cells (101, 102).

Because of these problems, the function of tumor specific T cells is limited by local factors within the tumor milieu. Therefore, modulation of this milieu may overcome tumor resistance to immunotherapy. By understanding the mechanisms of cancer cell immune-escape, it may be possible to design rational approaches to engineered DC-based therapies to target immunosuppression or prolong the survival of activated tumor specific T cells in an attempt to cure cancer effectively.

1.5.5.1 Interlukin-10 (IL-10) and regulatory T cells (Treg)

Within the tumor milieu, tumor cells and surrounding host stromal cells can secret immunosuppressive molecules, such as transforming growth factor-β (TGF- β), IL-10,

prostaglandin E2, and vascular endothelial growth factor (VEGF). Tumor-associated DCs usually have a low allostimulatory capacity, particularly if isolated from the progressing metastatic lesions, such as in malignant melanoma, or from blood, such as in patients with advanced breast cancer. By releasing cytokines such as IL-10, macrophage colony stimulating factor (M-CSF), and VEGF, tumors can prevent differentiation and function of APCs (103). Moreover, the suppressive tumor environment is also controlled by some immunosuppressive cells, such as Treg cells (104). Treg cells, as the professional immune suppressor cells, have been frequently found inside tumors and result in tumor escape from activated lymphocytes. Immune-suppression is related to tumoral IL-10 expression and recruitment of Treg cells.

IL-10 has been identified as a key immunomodulatory cytokine that is able to mediate immunosuppressive effects in the immune system. It plays a central role in maintaining the proper balance between protective immunity against infections/tumors and limiting proinflammatory responses to self-antigens. It inhibits $CD4^+$ T cell proliferation and reduces DC immunogenicity, with down-regulated expression of MHC II, ICAM-1 and CD80. IL-10 leads to the formation of T-cell anergy (105) and induces Treg cells that suppress antigen-specific T-cell responses (101, 106). The immunosuppressive level of IL-10 can be detected in tumor cell cultures of many human tumors (99, 107). It has been reported that tumor-induced IL-10 inhibit tumor-specific $CD8^+$ CTL cytotoxicity, resulting in the suppression of antitumor immunity and inhibition of protective immunity against the tumor (108). IL-10 also can impair the ability of DCs to stimulate $CD4^+$ T cell responses, indicating that this cytokine is capable of blocking tumor-specific Th1

responses (100). Moreover, IL-10 supports tumor growth by inhibition of macrophage function and induction of tumor and vascular cell proliferation in animal tumor models (109, 110). In the clinic, the level of IL-10 detected in the serum of patients becomes a negative indicator for clinical outcome of cancer therapy (111, 112). Therefore, T-cell suppression derived from tumor-secreted IL-10 becomes one of the major barriers to T-cell immunotherapy of tumors.

$CD4^+$ Th cells had been grouped into Th1 (IFN-γ producing cells that respond to intracellular pathogens), Th2 (IL-4-, IL-5- and IL-13-producing cells that respond to parasitic infections), Th17 cells (related to the development of autoimmune diseases) (113) and Treg cell (the suppressive T cells) (114) subsets. The third and the fourth subsets of Th cells, Th17 cells and Treg cells have emerged in the last few years. Both TGF-β and IL-6 can cause naïve T cells to differentiate into Th17 cells, but TGF-β alone guides naïve $CD4^+$ cells toward the suppressor Treg group (115, 116). Treg cells are responsible for inducing and maintaining peripheral tolerance (117). Treg cells have been associated with human tumors, and increased numbers of Treg cells have been reported in the blood and tumors of patients with various cancers (117). The induction of Treg cells is relevant to a number of tumor environmental factors, such as VEGF, IL-10, TGF-β, and indoleamine 2, 3-dioxygenase (IDO). Treg cells can inhibit tumor-specific cellular immune responses in a variety of ways. Treg cells ($CD4^+CD25^+$) expressing the IL-2 receptor, CD25, uptake IL-2 in the tumor microenvironment. IL-2 is not necessary to efficiently activate Treg cells, but is needed during suppression of responder cells, as specific blocking of the IL-2 receptor on Treg cells leads to a complete loss of regulatory

activity. In addition, IL-2 primes Treg cells to produce suppressive cytokine IL-10 upon re-stimulation (118). Human Treg cells are capable of directly killing T cells and APCs via the perforin or granzyme B pathways (119). CTLA-4^+ Treg cells can induce IDO expression in APCs, which can suppress T cell activation by depleting tryptophan (120). Treg cells can release IL-10 and TGF-β *in vivo*, and they directly inhibit T cell activation and suppress APC function by inhibiting the expression of MHC molecules, CD80, and CD86 and the production of IL-12 (117). Treg cells also induce B7-H4, a molecule of the B7 family and expression on APCs, which inhibit T cell activation. In addition, in a murine system, Treg cells were able to trigger high levels of IL-10 production by APCs, which up-regulated B7-H4 and induce an immunosuppressive response (121-124). Thus, Treg cells may inhibit TAA-specific immunity both directly and indirectly via APCs.

1.5.5.2 Short Lifespan of DC-activated $CD8^+$ T cells

The *in vivo* $CD8^+$ T cell responses include three main phases (125): (i) a proliferation phase of growth and differentiation of naive $CD8^+$ T cells into effector T cells, (ii) a contraction phase of transition between the large population of effector T cells to a smaller population (≈5-10%) of Tm cells (126), and (iii) the memory phase of long-term maintenance of memory T (Tm) cells in the host. The DC vaccine- activated $CD8^+$ T cells undergo a similar contraction phase of high-level activation-induced cell death (AICD). If most of the activated $CD8^+$ T cells die quickly due to the AICD via apoptosis, the therapeutic efficacy of DC-based immune therapy is greatly limited. Therefore, a

critically important issue in DC-based therapy is how to promote increased survival of activated T cells.

1.5.5.3 The Solid Tumor Environment Inhibit T Cell infiltration

In clinical trials, scientists found that immune therapy is still limited to treatment of early stage cancers, and that this therapy showed little therapeutic efficacy for large tumors. This is due to the solid tumor microenvironment, which inhibit the infiltration and activities of CTLs (127).

It has been reported that there is high interstitial pressure within a solid tumor. Elevated interstitial pressure principally reduces the driving force for extravasations of fluid and macromolecules in tumors, and also leads to an experimentally verifiable, radially outward convection which opposes the inward diffusion. Therefore, it could become a physical barrier, resulting in poor penetration of cytokine and immune cells into tumors (128).

In solid tumors, the expression of some factors that are related to T lymphocyte migration from blood to the tumor site is reduced or absent. In the antitumor response, when adhesion receptors such as L-selectin and the alpha 4β7 integrin on T lymphocytes bind to ligands expressed on the venular endothelium, the migration can occur. It has been reported that the ligands for L-selectin and alpha 4β7 were not expressed in endothelial venule of solid tumors, which inhibits lymphocytic infiltration to limit the efficiency of antitumor immunity (129). Vascular adhesion protein 1 (VAP-1) is also essential for T-cell adherence to, and migration across the endothelium (130). In solid

tumors, the level of VAP-1 was reduced (131). Thus, down-regulating the expression of VAP-1 in tumor vasculature can possibly impair the effectiveness of lymphocyte therapy. Vascular cell adhesion molecule (VCAM)-1/ ICAM-1 is another factor that causes lymphocyte adhesion to the tumor endothelium. They show low expression in solid tumors (132) and this low level of VCAM-1/ICAM-1 expression is related to the secretion of angiogenic molecules such as basic fibroblast growth factor (BFGF) (133, 134). The characterization of the solid tumor microenvironment explains some of the failure observed with immune cell based therapy.

1.6 Interlukin-6 (IL-6)

Interlukin-6 (IL-6) is a pleotropic cytokine and is secreted by many different cells, including monocyte/macrophages, fibroblasts, endothelial cells, keratinocytes, mast cells, T cells, dendritic cells and many tumor cell lines (135). The events causing an inflammatory response can increase IL-6 secretion. The location of the IL-6 gene in the human gene is 7p21 (136) and the transcription of the IL-6 gene requires several transcription factors, including nuclear factor κB (NF-κB), CAAT/enhancer-binding protein (C/EPB) family members and activator protein 1 (AP-1) (137). Moreover, the IL-6 promoter is inhibited by p53 and retinoblastoma (Rb) (138, 139). The molecular weight of IL-6 is between 21.5 and 28 kilo-dalton (Kd).

1.6.1 IL-6 signaling Pathway

The IL-6 receptor (IL-6R), as a member of the class I cytokine receptor family, contains ligand biding components (IL-6Rα) and signal transducing components (gp130) (140).

The IL-6Rα binds IL-6 directly and comprises two forms, a membrane-bound form and a soluble form (141). A homodimer of gp130, which is in the cell membrane, can not bind the IL-6, but plays an important role in the formation of a high-affinity IL-6 binding site and transduction of the IL-6 signal (142). Moreover, there is some soluble gp130 in human serum, but it is an antagonist of the IL-6/sIL-6Rα complex (143). IL-6 binding to a cell via the IL-6R (IL-6Rα/gp130 complex) triggers a series of events that leads to the activation of the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT3) pathway or the activation of mitogen-activated protein kinase (MAPK) pathway, switching on target genes (144).

1.6.2 The Function of IL-6 in Immune System

A large number of cells in the immune system are capable of IL-6 synthesis. IL-6 plays a key role in the progression from the initial innate immune response to infection to an adaptive immune response (145). IL-6 can activate the JAK/STAT3 pathway. The activated STAT3 dimerizes via phosphorylation and travels to the nucleus, triggering a transcriptional program (144). The function of this program is to promote growth and differentiation and prevent apoptosis (146). It has been reported that the IL-6 exerts an antiapoptotic effect on a wide variety of cells, including plasma cells, liver cells, neurons, master cells, endothelial cells and pancreatic β cells (147-151). In particular, it plays a central role in determining the number of memory and/or effector $CD4^+$ T cells in response to immunization over extended periods (152). Moreover, it has been determined that IL-6 inhibits apoptosis of activation-induced cell death of T cells through reduction

of the level of T cell receptor (TCR)/CD3-induced apoptosis and expression of Fas/ Fas ligand (FasL). Furthermore, in this process, IL-6 can not decrease IL-2 induced activation of T cells (153).

Moreover, as mentioned in Section 5.5.1, Treg cells inhibit TAA-specific immunity both directly and indirectly through APCs. IL-6 can inhibit the formation of $CD4^+$ 25^+ Treg cells in the immune system (115, 116). Therefore, IL-6 may increase T-cell activation and overcome the immune-suppression that is induced through tumoral IL-10 expression and Treg cells. IL-6 may be a candidate for an engineered-cancer vaccine.

1.7 Adenoviruses (AdVs)

In the 1953, adenovirus (AdV) was first discovered and the features have since been broadly characterized. Several of these features have made adenovirus an excellent vehicle for gene transfer and transgene expression in mammalian cells.

1.7.1 Adenovirus Properties

AdV have been isolated from several avian and mammalian cells. Of these, more than 40 known serotypes of human AdV have been divided into 6 subgroups (A to F), according to their oncogenic potential in rodents and their similarity in DNA sequences (154). The serotype 2 and the serotype 5 of subgroup C have been developed as vectors for gene delivery.

AdV, a non-enveloped icosahedral nucleocapsid, is composed of 240 hexons and 12 pentons (155). The penton subunits and the hexons build up the twelve vertices of the

icosahedrons and the face of the icosahedral capsid, respectively. There is fiber protein projecting from each of the penton, and at the end of the each fiber, there is a globular tip named the knob domain. The AdV contains a linear double stranded DNA genome, the size of which is between 30 to 40 kb. The serotype 5 genome is 35,935 base pairs (bp) (156).

AdV transcription consists of two phases, early and late, which happen before and after viral DNA replication, respectively. Based on these, the AdV genome is divided into two kinds of genes, early transcribed regions(E1, E2, E3, E4) and late transcribed regions (L1, L2, L3, L4, L5). The early transcribed regions play an important role in adenoviral gene transcription, DNA replication, host immune-suppression and inhibition of host cell apoptosis. The E1 gene is essential for the assembly of infectious virus particles. The gene products of the E2 region are related to viral DNA replication and the ensuing transcription of late genes. Moreover, the proteins encoded by E4 are involved in the metabolism of viral messenger RNA, provide functions that promote viral DNA replication and shut-off of host protein synthesis. However, the E3 proteins are involved in evading host immunity, which is not essential for viral growth in vitro. After the replication of viral DNA, the expression of the early genes is stopped and the late genes start transcribing via the major late promoter. The late genes encode for the formation of the virion's structural proteins (157).

1.7.2 Adenovirus Infection

For the subgroup C AdV, including serotypes 5 and 2 AdV, during the process of Ad infection, the first step is the binding of AdV on target cells, which requires two specific cell-surface receptors, "Coxsackie virus and Adenovirus receptor" (CAR) and αvβ3 or αvβ5 integrins. First, the knob domain located at the end of the AdV fiber binds to the CAR on the target cell (158, 159). After the attachment step, interaction between the arginine-guanine-aspartate (RGD) modif located on the penton base and the αvβ3 or αvβ5 integrins leads to the internalization of the virus through endocytosis (160). It has been reported that the DCs do not express CAR but high levels of αvβ3 or αvβ5 integrins (161-163). To improve infection of AdV into CAR-negative (or low) cell lines, an RGD peptide motif was incorporated into H1 loop of the AdV knob domain. It has been suggested that the efficiency of DC transduction by fiber-modified AdV is enhanced about 10-fold *in vitro* and there is a 5-10 fold increase of transgene expression in the fiber-modified AdV transduced DCs (164-166). The fiber-modified AdV vector targeted DCs may be practical in the development of AdV-based vaccines.

Once entering the target cell, the virus releases the endosome into the cytosol via penton base mediating lysis of the endosomal membrane. Then, the viral DNA enters to the nucleus and transcription begins. In the nucleus of the infected cell, transcription, replication and viral packaging will occur.

1.7.3 Recombinant Adenovirus

Adenovirus has been adapted to make it a versatile tool for gene delivery and gene therapy. Recombinant adenovirus (rAdV) has had the E1 and E3 domains deleted. The E1 gene deletion prevents the rAdV from replicating itself and therefore no cell lysis occurs. The E1 gene is complemented by an adenovirus packaging cell line (e.g. AD-293). The E3 gene is dispensable for viral growth. These two deletions also allow for an additional 7.5 kb of foreign DNA (167).

Compared with the other vectors, rAdV vector has the following advantages: (i) AdV can infect a wide range of host species *in vitro* and *in vivo* and express human and non-human proteins. AdV also has low pathogenicity in human and no significant side effects (168). (ii) AdV can infect and express genes in both replicative and non-replicative cells, however, the retrovirus can only infect replicative cells. (iii) AdV is relatively stable and can be obtained in high titer (e.g. 10^{11}-10^{12} plaque-forming units (PFU)/ml). (iv) AdV is capable of accepting large-sized gene (7.5kb) insert and expressing multiple genes in the same cell line or tissue. (v) AdV does not integrate into the host chromosome and the inserted genes are expressed epichromosomally (169). This prevents the possibility of disturbing the host cellular genes and does not cause the host gene to be mutated. (vi) AdV can propagate in suspension cultures, allowing for easy scale-up of production (59).

Chapter 2

HYPOTHESIS

It is recognized that dendritic cells (DCs) play an important role in stimulating antitumor immunity. Based on these powerful important roles of DCs, DC-based vaccines have been proposed as a treatment modality for cancer. In animal models, vaccination with DCs pulsed with tumor peptides, lysates, mRNA or pulsed with apoptotic/ necrotic tumor cells, or DCs engineered with transgenes, including tumor-associated antigens (TAAs) and immunomodulatory proteins, could induce significant antitumor cytotoxic T lymphocyte (CTL) responses and antitumor immunity. However, in clinical trials, only a small part of patients have responded to these DC-based vaccines (170, 171). In recent years, some problems with DC-based vaccines have been identified, in addressing which will eventually lead to improve the feasibility and efficacy of the approach in years to come. Among these problems, the most significant include (i) immune suppression related to tumoral interleukin (IL)-10 expression (99, 100) and recruitment of regulatory T (Treg) cells (101, 102); and (ii) the short life span of activated T cells due to high-level of activation-induced cell death (AICD). It is shown that the IL-6 cytokine can inhibit $CD4^+CD25^+$ Treg cell-mediated immune suppression (115, 116) and enhance the activated T cell survival via suppression of AICD without interfering with the process of T cell activation (153). The AdV vector is known to be a good candidate to introduce gene into DCs and could play a beneficial role in the development of this treatment.

With this knowledge in mind, we hypothesize that: (i) DCs infected with the IL-6 gene via adenovirus can secrete transgenic IL-6; (ii) $CD8^+$ CTL cells activated by the IL-6 infected DCs may counteract Treg-induced immune suppression; (iii) $CD8^+$ CTL cells activated by IL-6 infected DCs may show prolonged survival.

Therefore, IL-6 infected DC-activated $CD8^+$ CTL, which have prolonged survival and enhanced counteraction of Treg-mediated immune suppression, could greatly increase immunogenicity, and lead to efficient $CD8^+$ CTL responses and antitumor immunity.

Chapter 3

OBJECTIVES

The aims of this thesis were to study (i) the effects of IL-6 in DC-based vaccines and (ii) the immune mechanism of these effects induced by IL-6. To reach our aims, we plan to (i) construct the adenovirus (AdV) with an IL-6 transgene insertion for use as a vector to introduce the IL-6 gene into DCs, (ii) generate the IL-6 transgene-engineered DC vaccine expressing IL-6, (iii) assess weather the IL-6 engineered-DC vaccine can enhance $CD8^+$ CTL responses and antitumor immunity in a BL6-10_{OVA} animal tumor model, and (iv) study the immune mechanism underlying the results of the IL-6 engineered-DC vaccine.

3.1 Construct AdV Vector

To transfer the IL-6 gene to DCs, we will construct a fiber-modified AdV vector with an IL-6 gene insert using the AdEasy system. Since DCs do not express 'Coxsackie virus and Adenovirus receptor' (CAR) but rather high level of integrin (161-163), the fiber-modified AdV vector can enhance the infection of DCs by the AdV.

3.2 Generate the IL-6 Transgene-engineered DC

For studying the effect of DC vaccine secreting IL-6 cytokine, we will transfect bone-marrow derived DCs with AdV containing IL-6 gene insert. These AdV-mediated IL-6 transgene engineered-DCs should express the high level of IL-6.

3.3 Assessing Weather the IL-6 Engineered-DC Vaccine can Enhance $CD8^+$ CTL Responses and Antitumor Immunity

After being pulsed with ovalbumin (OVA) antigen, IL-6 infected DCs become the IL-6 engineered-DC vaccine. The effect of IL-6 engineered DC-based cancer vaccine will be examined by analyzing (i) the effect of $AdV_{IL\text{-}6}$ infection on DC phenotype and function, and (ii) the antitumor effect of this vaccine in a BL6-10_{OVA} animal tumor model.

3.4 Elucidating the Immune Mechanism underlying the Results of IL-6 Engineered-DC Vaccine

To explore the possible mechanism underlying the effects induced by the IL-6 engineered-DC vaccine, we will (i) generate the IL-6 engineered DC-activated $CD8^+$ T cells *in vitro*, (ii) assess the phenotypic characterization of these T cells by flow cytometry, (iii) determine weather these T cells show prolonged survival, and (iv) analyze the therapeutic effect of these T cells in mice bearing solid EG7 tumor.

Chapter 4

MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents and Suppliers

In the table 4.1, some reagents used in the experiments presented in this thesis are displayed. All of the reagents used are of the molecular biology or research grade. In the table 4.2, all of the commercially available kits used in this study are displayed.

Table 4.1: List of reagents and suppliers

Reagent	Supplier Name
Agar	Invitrogen
Agarose	Invitrogen
Alkaline phosphatase	New England Biolabs
Ammonium chloride	EM Sciences
Ampicillin	Sigma
Bacto-tryptone	BD Bioscience
BSA	Sigma
Calcium chloride	Sigma
Cesium chloride	Sigma
CFSE	Molecular Probes
Chloroform	EM Sciences
RPMI 1640	Invitrogen
DMEM	Invitrogen
AIM-V medium	Invitrogen

Reagent	Supplier Name
DMSO	Sigma
dNTP mix (dATP, dCTP, dGTP, dTTP)	Invitrogen
Electroporation cuvette (0.2cm, 0.4 cm gap)	Bio-Rad
EMEM	Invitrogen
Ethanol	EM Sciences
Ethidium bromide	Sigma
FBS	Hyclone
HEPES	Invitrogen
Formalin	EM Sciences
Gentamicin Reagent Solution	Invitrogen
Glutaraldehyde	Sigma
Glycerol	BDH Inc
Glycine	EM Sciences
GM-CSF	R&D Systems
Hydrochloric acid	EM Sciences
IL-2	Peprotech
IL-4	R&D Systems
Isopropanol	EM Sciences
Kanamycin	Sigma
Lysozyme	Sigma
Magnesium chloride	Sigma
Methanol	EM Sciences
Non-fat dry milk	Carnation
PEG-8000	Sigma
Phenol	EM Sciences
RNase	Amersham Biosciences
RPMI 1640	Invitrogen
SDS	Sigma/Bio-Rad
Sodium acetate	BDH Inc
Sucrose	BDH Inc
Sulfuric Acid (H_2SO_4)	BDH Inc
T4 DNA ligase	Invitrogen

Reagent	Supplier Name
Taq DNA polymerase	Invitrogen
TRIS	EM Science
Trypan Blue Stain	Invitrogen
Trypsin/EDTA	Invitrogen
Tween 20	Bio-Rad
X-gal	Invitrogen
Yeast Extract	Difco
β-mercaptoethanol	Bio-Rad
λDNA/*Hind*III marker	Invitrogen
φX174/*Hae*III fragment marker	Invitrogen
Ovalbumin (OVA)	Sigma
OVA I peptide	Sigma

Table 4.2: Commercially available kits used in this study

Commercial Kits	**Supplier Name**
Superscript first strand synthesis for RT-PCR kit	Invitrogen
GenElute Agarose Spin column	Sigma
PE-labeled H-2K^{b}/OVA $_{257-264}$ tetramer	Beckman-Coulter
Plasmid Mini Kit	Qiagen
Qiaprep Spin Kit	Qiagen
Mouse IL-6 ELISA Set	BD Bioscience
TMB Substrate Kit	BD Bioscience
TA Cloning Kit	Invitrogen
Zero Blunt PCR cloning kit	Invitrogen
Nylon wool column	C&A Scientific
Anti-mouse CD4 (L3T4) paramagnetic beads	Invitrogen
Cytofix/ CytoPerm Plus with GolgiPlug Kit	BD Bioscience
Slide-A-lyzer dialyzing cassette	Pierce

4.1.2 Antibodies

A variety of Abs used in our study and their respective suppliers are listed in the table 4.3. These Abs were conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or biotin.

Table 4.3: List of antibodies and respective suppliers

Antibody	Supplier
Biotin conjugated anti-mouse MHC class II I_a^b	BD Bioscience
Biotin conjugated anti-mouse CD_{11c}	BD Bioscience
Biotin conjugated anti-mouse CD25	BD Bioscience
Biotin conjugated anti-mouse CD40	BD Bioscience
Biotin conjugated anti-mouse CD54	BD Bioscience
FITC conjugated anti-mouse CD8	Beckman-Coulter
Biotin conjugated anti-mouse CD8	BD Bioscience
Biotin conjugated anti-mouse CD80	BD Bioscience
Biotin conjugated anti-mouse CD69	BD Bioscience
PE conjugated anti-mouse CD62 ligand (CD62L)	BD Bioscience
PE conjugated anti-mouse Fas ligand (FasL)	Blue Genes
FITC conjugated anti-mouse Perforin	ALEXIS
FITC conjugated streptavidin	BD Bioscience

4.1.3 Cell Lines

A murine dendritic cell line DC2.4 was obtained from K. Rock (Dana-Farber Cancer Institute, Boston, Mass) and cultured in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) supplemented with L-glutamine (2 mM), non-essential amino acids (100 μM), 2-mercaptoethanol (2-ME, 50 μM), 1% (v/v) 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES; Invitrogen, Burlington, ON, Canada) and 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT). The mouse OVA-specific B16 melanoma cell

line BL6-10_{OVA} was generated in our laboratory (172). The OVA transfected cell line EG7, which is derived from the mouse thymoma cell line EL4, was obtained from American Type Culture Collection (ATCC). Both the BL6-10_{OVA} and EG7 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Burlington, ON, Canada) containing 10% (v/v) FBS (Hyclone, Logan, UT) and G418 (0.5mg/ml). The human embryonic kidney cell line 293 containing the adenoviral E1 genes were purchased from Microbix (Toronto, ON, Canada) and cultured in Minimum Essential Medium containing Earle's Salts (EMEM; Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS. All of the media used also included 30 μg/ml gentamicin solution (Invitrogen, Burlington, ON, Canada).

DC2.4, BL6-10_{OVA} and 293 cell lines were adherent cell lines. The DC2.4 and BL6-10_{OVA} cell lines were passaged using Trypsin/ethylenediamine tetracetic acid (EDTA; Invitrogen, Burlington, ON, Canada) whereas the 293 cell line was passaged using a 1× citric saline solution [10% (w/v) KCl and 4.4% (w/v) sodium citrate]. All cell lines were cultured in a 37°C humidified CO_2 incubator with a 5% CO_2 atmosphere. Trypan-Blue (Invitrogen, Burlington, ON, Canada) was used for cell counting on a haemocytometer.

4.1.4 Animals

The OVA-specific T cell receptor (TCR) transgenic OTI mice, having a transgenic Vα2Vβ5 TCR specific for the $OVA_{257–264}$ epitope in context of H-$2K^b$ (173), and naïve C57BL/6 (B6, CD45.2^+) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were approximately 4-6 weeks old and housed in the Saskatoon Cancer

Centre animal facility. All animal experiments were carried out in accordance with the Canadian Council for Animal Care guidelines.

4.1.5 Bacterial Cells

DH5α *E. coli* and *E. coli* BJ5183 cells were used as the bacterial hosts for vector propagation and for homologous recombination in the construction of AdV vectors, respectively. *E. coli* BJ5183 cells were obtained from Stratagene (La Jolla, CA). Both of these bacteria were grown in medium containing Lauria-Bertani (LB) broth consisting of 1.0% (w/v) tryptone (Voigt Global Distribution, Lawrence, KS), 0.5% (w/v) yeast extract (Voigt Global Distribution, Lawrence, KS) and 1.0% (w/v) NaCl complemented with the appropriate antibiotic such as ampicillin (100μg/ml) or kanamycin (50 μg/ml), depending on the vector, in a shaking incubator at 37°C. For isolation of transformed bacterial cells, the cells were plated onto selective LB-agar plates, containing LB broth with 1.5% (w/v) agar complemented with the appropriate antibiotic, and incubated at 37°C overnight.

4.2 Methods

The routine molecular biology protocols used in our study were based upon Molecular Cloning: A Laboratory Manual by Sambrook and colleagues and Sambrook and Russel (174, 175).

4.2.1 RNA Methods

4.2.1.1 RNA Isolation

The RNA was isolated from the cell samples by using the RNeasy mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's suggested protocol. Isolated RNA was re-suspended in RNase free distilled water and stored in liquid nitrogen until needed. The concentration and purity of the isolated RNA were measured by standard A_{260}/A_{280} spectrophotometric readings.

4.2.1.2 cDNA Synthesis and RT-PCR

The isolated RNA was used as a template for the first-strand cDNA synthesis using the SuperScript First-strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen, Burlington, ON, Canada) in accordance with the instructions provided by the manufacturer. In brief, 5 µg of RNA was incubated in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl2, 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 0.5 µg Oligo(dT)12-18, 40 units of RNase inhibitor and 50 units SuperScript II reverse transcriptase enzyme, for a final volume of 20 µl. To terminate the reaction, the sample was incubated at 42°C for 50 min followed by 70°C for 15 min. The sample was then treated with 1 unit of RNase H to remove remaining RNA. The cDNA samples were used either immediately for polymerase chain reaction (PCR) reactions or stored at -20°C for future use.

The PCR reactions were done in a volume of 100 µl containing the cDNA sample, 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 1.5 mM MgCl2, 1 mM deoxynucleotide triphosphate (dNTP) mix (dATP, dCTP, dGTP, dTTP), 10 ng of each primer and 2.5 units of Taq polymerase (Invitrogen, Burlington, ON, Canada). As shown in the table 4.4, the PCR primers for the IL-6 gene and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed based on the gene sequence obtained by GeneBank Accession number NM_031168 and NM_008084, using MacVector software, respectively. The level of GAPDH expression was used as a control reaction for RT-PCR samples. The PCR cycle included the initial DNA denaturing step at 94°C for 2-5 min followed by 30 amplification cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec followed with a final termination step for 7-10 min at 72°C. One-tenth of the reaction volume was resolved by 1.5% agarose gel electrophoresis after PCR amplification.

Table 4.4: RT-PCR primers

Gene	Template	Forward and reverse primers
IL-6	DC2.4 RNA	5'- ACCGC TATGA AGTTC CTCTC TGC -3' 5'- AGGCA TAACG CACTA GGTTT GC -3'
GAPDH	DC2.4 RNA	5'- CAGGT TGTCT CCTGC GACTT -3' 5'- CTTGC TCAGT GTCCT TGCTG -3'

4.2.2 DNA Methods

4.2.2.1 Restriction Enzyme Digest

The restriction enzymes used in this study were purchased from either GE Healthcare (Baie d'Urfé, Québec, Canada) or New England Biolabs (NEB, Pickering, ON, Canada). The restriction enzyme digestions were performed using at least 1μg DNA in 1× the final recommended buffer suggested by the manufacturer and at least 1 unit of the specified enzymes. For complete restriction enzyme digestion, the reactions were incubated at 37°C for 1 hour.

4.2.2.2 Agarose Gel Electrophoresis

Agarose gels were casted using a GelHorizon 58 Apparatus of varying agarose concentrations ranging from 0.7% to 1.5% in Tris-acetate EDTA (TAE) buffer [40 mM Tris-acetate, 1 mM EDTA containing 1 μg/ml ethidium bromide (EtBR; Sigma, Oakville, ON, Canada)]. The sample products were loaded onto the gels with gel loading buffer [0.042% (w/v) bromophenol blue, 6.67% (w/v) sucrose] along with the DNA markers, λDNA/Hind III and φX174/Hae III fragment markers (Invitrogen, Burlington, ON, Canada). Gel electrophoresis was performed between 90 to 110 V in TAE buffer for varying times to attain optimal resolution. The gel was visualized by UV illumination using a gel documentation system (Bio-Rad, Mississauga, ON, Canada).

4.2.2.3 Purifying Linear DNA Fragments

The GenElute Agarose spin columns (Sigma, Okaville, ON, Canada) were used to isolate and purify the linear DNA fragments from DNA agarose gels, according to the instructions provided by the manufacturer. Briefly, the spin column was pre-washed with 100ul of 1× Tris EDTA (TE) (10mM tris, PH 8.0, with 1mM EDTA). The band of DNA fragment was then cut from the agarose gel, sliced into smaller pieces and placed onto the pre-washed spin column. The DNA solution was collected in a separate tube after centrifugation of column at maximum speed for 10 min. Following ethanol precipitation, the DNA pellet was re-suspended in distilled water or TE buffer, and then stored at -20°C until use.

4.2.2.4 Ligation of IL-6 PCR Products with pCR2.1 Vector

The ligation of IL-6 PCR products with pCR2.1 vector was performed using the TA cloning Kit (Invitrogen, Burlington, ON, Canada). The Taq polymerase has terminal transferase activity that adds a single deoxyadenosine (A) overhang to the 3' ends of PCR products. The pCR2.1 vector supplied in the kit is linearized and has single 3' deoxythymidine (T) overhangs. Therefore, it is possible to clone Taq-generated PCR products directly into a linearized cloning pCR2.1 vector. Ligations were performed using T4 DNA ligase overnight in a 14°C water bath.

4.2.2.5 Sequencing IL-6 PCR Products

The RT-PCR products of IL-6 in TA vectors were sequenced with the M13 forward (-20), 5'- GTAAA ACGAC GGCCA G -3', and reverse, 5'- CAGGA AACAG CTATG AC -3' primers, at the Plant Biotechnology Institute of the National Research Council of Canada in Saskatoon, Saskatchewan.

4.2.2.6 Competent Cells and Transformation

4.2.2.6.1 Competent Cell Preparation and Chemical Transformation

Competent cells were prepared based on a standard method previously described by Chan *et al* (176). Briefly, an overnight culture of bacterial cells were grown in LB medium until mid log-phase growth with an OD_{600} reading between 0.4-0.6, then spun down. The cell pellet was washed twice with fresh 50 mM Calcium Chloride (CaCl) 10 mM Tris-HCl (pH 8.0) and then re-suspended in the same solution. Transformations were performed incubating 50-100 µl of competent cells with the DNA mixture on ice for 30 min. The sample was heat-shocked by quickly placing the sample in a 42°C water bath for 50 seconds, then immediately returning it to ice for 1 min. To allow the bacteria to recover before plating the cells on selective LB-agar plates, SOC media [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast-extract, 0.05% (w/v) NaCl, 20 mM glucose] was added and the sample was incubated at 37°C for 1 hour. When blue and white colony screening was needed, the selective LB-agar plates were supplemented with 50µg/ml X-gal (Invitrogen, Burlington, ON, Canada) and 0.1 nM isopropylthio-β-D- galactoside (ITPG; Invitrogen, Burlington, ON, Canada).

4.2.2.6.2 Electrocompetent Cell Preparation and Eletrotransformation

Electrocompetent cells were prepared as previously described (175, 177). The bacterial cells were grown overnight in selective LB broth. In the following morning, these bacterial cells were sub-cultured in fresh media until mid-log phase was reached with the OD_{600} reading between 0.4-0.6. Bacterial cells were chilled then centrifuged at 1,000 × g in a JA-10 rotor (Beckman, Mississauga, ON, Canada). After washing the sample twice, the sample was re-suspended in a 10% (v/v) glycerol in water solution and stored at -80°C until required. Both the bacterial cells and the DNA were placed into chilled electroporation cuvettes (0.2 cm gap; Bio-Rad, Mississauga, ON, Canada) and then electroporated using the conditions: 200 Ω (ohms), 2.5 kV and 25 μFD using a Gene Pulser with Pulse Controller (Bio-Rad, Mississauga, ON, Canada). To allow the bacteria to recover before plating the cells onto selective LB-agar plates containing the appropriate antibiotic, LB media was added to the sample and incubated at 37°C for 1 hour.

4.2.2.7 Isolation of Plasmid DNA from Host Bacterial Cells

4.2.2.7.1 Isolation of Plasmid DNA from Host Bacterial Cells (mini-prep)

To screen colonies for positive transformants, a small scale plasmid preparation using an alkali lysis method was performed. Isolated colonies were selected and cultured overnight in LB with the appropriate antibiotic. In the following morning, the cultured cells were re-suspended in 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 2 mg/ml lysozyme (Sigma, Oakville, ON, Canada), then lysed with a 200 mM NaOH, 1%

sodium dodecyl sulfate (SDS) solution followed by the addition of 3 M sodium acetate, pH 5.2. This method precipitated out the bacteria chromosomal DNA, cellular protein and debris. The supernatant was extracted twice with phenol:chloroform:isoamyl alcohol followed by an ethanol precipitation. The precipitated DNA was re-suspended in TE (pH 8.0) containing 40 μg/ml RNAse A. DNA concentration was measured by standard A_{260}/A_{280} spectrophotometric readings and visualized on agarose gels.

4.2.2.7.2 Isolation of Plasmid DNA from Host Bacteria Cells (large scale)

Bacterial cells were grown overnight in Terrific broth (TB) [1.2% (w/v) bacto-tryptone, 2.4% (w/v) yeast-extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4 and 72 mM K_2HPO_4] in a shaking incubator at 37°C. The sample was treated with the same solutions as the mini-prep with the addition of an extra polyethylene glycol (PEG) purification step. Finally, the purified DNA was re-suspended in TE (pH 8.0) or in sterile phosphate buffered saline (PBS) for use in infection. The concentration of the purified DNA was determined using standard A_{260}/A_{280} spectrophotometric readings and visualized on agarose gels.

4.2.3 AdV Methods

4.2.3.1 AdV Vector Construction

The vectors pShuttle-CMV and pAdEasy-1 were obtained from Dr. Lixin Zhang (John Hopkins/Sidney Kimmel) and Stratagene (La Jolla, CA), respectively. To increase the effect of AdV infection of DC, the fiber-modified adenoviral vector, AdV(RGD), was required. The fiber gene was modified by inserting the arginine-guanine-aspartate (RGD)

motif into the viral HI loop and the fiber-modified adenoviral vector, AdV(RGD), was created using the AdEasy system (178) similar to the method described by Liu and colleagues (179) as described below.

To create the fiber-modified vector, the pAdEasy-1 vector was digested with *Bam*HI, resulting in 21.7- and 11.7-kb DNA fragments. The 11.7-kb fragment was inserted into pCRBlunt vector, which is from Zero Blunt PCR cloning kit (Invitrogen, Burlington, ON, Canada), to generate pCR11.7 vector. This pCR11.7 vector was digested with *Eco*RI, resulting in 9.3- and 5.6-kb DNA fragment. The 9.3-kb fragment, consisting of the pCRBlunt backbone, underwent self-ligation, constructing pCR9.3 vector. PCR amplification was utilized to replace sequences within the *Stu*I-*Afl*II sites of the fiber knob domain in pCR9.3 vector. In the PCR, the sense primer 1 (5'- CAACA AAGGC CTTTA CTTGT TTACA GCTTC A -3') and antisense primer 2 (5'- TGACA TAGAG TACTG GTTTA GTTTT GTCTC CGTTT AAG -3') were utilized to amplify a 680-base pair (bp) P1+P2 fragment (nucleotides 31950-32630 of the Ad5 genome), while the sense primer 3 (5'- ACTAA ACCAG TACTC TATGT CATTT TCATG GGACT GGT -3') and the antisense primer 4 (5'- TGGAC AGCGA CATGA ACTTT AAGTG AGCTG -3') were utilized to amplify a 435-bp P3+P4 fragment (nucleotides 32690-33125 of the Ad5 genome). The P1+P2 and P3+P4 fragments were gel purified, mixed, and joined by PCR using P1 and P4, resulting in a 1.1-kb fragment. This fragment contains part of the knob sequence of Ad5 with *Afl*II (nucleotide 31950) and *Stu*I (nucleotide 33125) sites. A deletion was created from 32631-32689 removing amino-acid residues VTLTI TLNGT QETGD TTPSA, and incorporated a single mutation from T to A to create a *Sca*I site in

the HI loop. Plasmid pCR9.3(AS) was formed by inserting the 1.1-kb PCR fragment into the pCR9.3 vector. A duplex was constructed by two annealing complimentary oligonucleotides (5'- AACAC TAACC ATTAC ACTAA ACGGT ACACA GGAAA CAGGA GACAC AACTT GCGAC TGTAG AGGAG ACTGC TTTTG TCCAA GTGCA T -3' and 5'- ATGCA CTTGG ACAAA AGCAG TCTCC TCTAC AGTCG CAAGT TGTGT CTCCT GTTTC CTGTG TACCG TTTAG TGTAA TGGTT AGTGT T -3'). This 86-bp duplex was cloned into *Sca*I-digested pCR9.3(AS), forming the pCR9.3(RGD) plasmid containing the Ad5 complete sequence (nucleotide 31950-33125) and an additional RGD-4C sequence, CDCRGDCFC, in the HI loop between nucleotides 32679 and 32680. The previously constructed 5.6-kb fragment was inserted into *Eco*RI-digested pCR9.3(RGD) to form pCR11.7(RGD). The resulting 11.7-kb *Bam*HI band from pCR11.7(RGD) was ligated into the previous 21.7-kb *Bam*HI fragment of pAdEasy-1, resulting in pAdEasy(RGD). This 33.4-kb plasmid contains the pAdEasy-1 sequence and an additional RGD-4C sequence in the HI loop. All of the insert orientations within vectors were determined by sequencing and restriction analysis.

The cDNA fragment of IL-6 (*Kpn*I/ *Eco*RV) from the pCR2.1-IL-6 vector was ligated into the pShuttle-CMV vector to form the adenoviral vector pShuttle-CMV-IL-6. DNA ligation was performed using 50 ng of vector DNA and at least 200 ng of purified insert DNA, in a 10 μl volume containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM Adenosine triphosphate (ATP) and 1-5 unit T4 DNA ligase (Invitrogen, Burlington, ON, Canada) in a 14°C water bath overnight.

Figure 4.1 shows an outline of the steps involved in constructed the recombinant fiber-modified adenovirus AdV_{IL-6} expressing the transgene IL-6 using the AdEasy(RGD) system. Briefly, pShuttle-CMV-IL-6 was digested with *Pme*I to linearize the shuttle vector. The linearized shuttle vector was treated with alkaline phosphatase, gel purified and ethanol precipitated. The purified DNA was spun down, dried and re-suspended in distilled water. A total of 1 μg of the PmeI-digested shuttle vector was then co-transformed into 100 ng of BJ5183 *E. coli* cells, which already contained the backbone vector pAdEasy(RGD), via electrotransformation (as outlined in Section 4.2.2.6.2). The transformed cells were plated on selective LB-agar plates containing 100μg/ml kanamycin overnight. In the following morning, a number of small colonies were selected and cultured in LB broth supplemented with 100 μg/ml kanamycin. The vector DNA was isolated by performing mini-preps from bacteria. The DNA was screened using restriction enzyme analysis and analyzed on a 0.7% agarose gel. This allowed for positive selection of the recombinant vector $pAdEasy(RGD)_{IL-6}$, which was further transformed into DH5α *E.coli* host bacterial cells using chemical transformation methods.

The $pAdEasy(RGD)_{IL-6}$ plasmid DNA was purified using plasmid mini kits (Qiagen, Mississauga, ON, Canada) and digested with *Pac*I, releasing a small fragment consisting of the kanamycin resistance gene and the origin of replication (ori). To comfirm that *Pac*I digestion was complete, electrophoresis was performed on a 0.7% agarose gel. The reaction was then cleaned via phenol:chloroform:isoamyl alcohol (25:24:1) extraction followed by an ethanol precipitation. A total of 5 μg of *Pac*I-digested $pAdEasy(RGD)_{IL-6}$ DNA was re-suspended in sterile water for use in 293 cells transfection using

Lipofectamine (Invitrogen, Burlington, ON, Canada), described below, to produce AdV$(RGD)_{IL-6}$, which is referred to as AdV_{IL-6}. AdV_{pLpA}, the control AdV containing no transgene, was previously created and readily available in Dr. Xiang's lab (180).

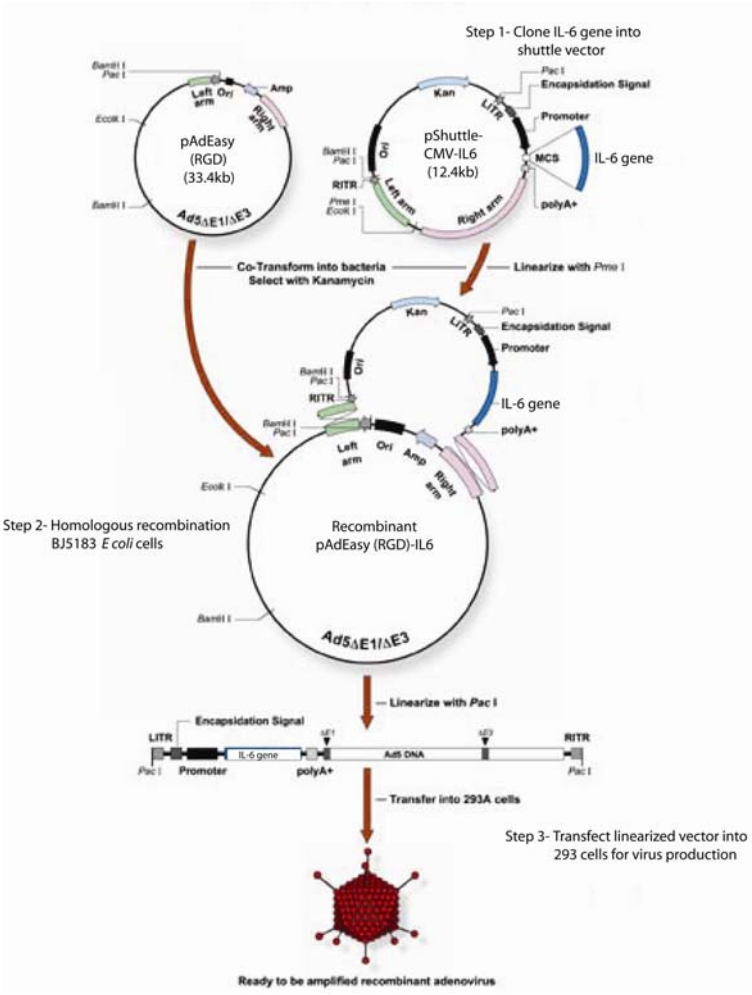

Figure 4. 1: Schematic overview of the AdEasy system.

The shuttle vector, containing the IL-6 gene, is linearized with PmeI restriction enzyme. The pShuttle-CMV-IL-6 and pAdEasy(RGD) are co-transformed into BJ5183 *E. coli* cells to allow homologous recombination between the two vectors, resulting in formation of pAdEasy(RGD)-IL-6. The resulting pAdEasy(RGD)-IL-6 was then infected into 293 cells for viral production of the replication-deficient recombinant AdV, AdEasy(RGD)-IL-6 (referred to as AdVIL-6).

4.2.3.2 Liposome Transfection

A liposome-based method was used for transfection of the *Pac*I-digested recombinant pAdEasy(RGD)$_{IL-6}$ DNA into 293 cells to produce the AdV$_{IL-6}$. The day before transfection, 293 cells were re-plated at a cell density of 2×10^6 cells per T25cm^2 flask. For transfections, 5 μg of *Pac*I-digested recombinant pAdEasy(RGD)$_{IL-6}$ was added to 20 μl of Lipofectamine in serum-free EMEM media and incubated at room temperature for 30 min. The DNA:liposomes complexes were added to the flasks and incubated at 37°C in a CO_2 incubator. After 4 hours, the serum-free EMEM media was changed to EMEM with 10% FBS. The flasks were continually monitored by microscopy for 7-10 days for plaque formation. To maintain ideal growth conditions, the medium was changed every 3 days. When the appearance and extension of cytopathic effects (CPE) were observed in the flask, the cells were harvested. The cell pellet was re-suspended in serum free EMEM and underwent five freeze/thaw cycles performed at -80°C and 37°C respectively, to prepare the initial crude viral lysate.

4.2.3.3 AdV Amplication and Purification

4.2.3.3.1 AdV Amplication

The initial crude viral lysate was amplified by infecting a large number of flasks of 293 cells. After infected cell culture pellets were harvested and had undergone five freeze/thaws cycles, the sample was spun down and the supernatant was harvested to further infect additional 293 flasks. The ration of infection during the amplification was one to five, which meant that the AdV derived from one T175cm^2 flask can infect 293

cells in five T175cm^2 flasks. Amplification continued until the number of the flasks of infected T175cm^2 flask reached to 24.

4.2.3.3.2 AdV Purification

After harvesting the infected flasks from the final amplification step, cells were subjected to at least five freeze/thaw cycles. To remove some of the cell debris and as much protein as possible, the sample was spun down at 3,000 rpm for 10 min and 10,000 rpm at 4°C for 15min. The recombinant AdV was then purified using a Double Cesium Chloride Gradient method. The supernatant was gently layered on top of a cesium chloride (CsCl) discontinuous gradient using Quick-Seal Centrifuge tubes for ultracentrifugation. The discontinuous layer, which removes the majority of cellular contaminants and defective viral particles, consisted of 1.25 gm/ml CsCl layered gently over a 1.40 gm/ml CsCl layer. All of the CsCl solutions were prepared using 1X TD buffer consisting of 140 mM NaCl, 5 mM KCl, 25 mM Tris and 0.7 mM Na_2HPO_4. Samples were spun in a Beckman ultracentrifuge at 45,000 rpm for 2 hours using a Type 80 Ti rotor at 20°C. The lower opalescent band was collected. To completely separate infectious from defective viral particles, the collected sample was placed onto a 1.34 gm/ml CsCl continuous gradient and spun at 45,000 rpm for 18 hours at 20°C, and the viral band was carefully aspirated. Finally, to remove the CsCl, the viral sample was placed into a Slide-A-lyzer dialyzing cassette (Pierce, Nepean, ON, Canada) and dialyzed against 10 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$ buffer solution for several hours at 4°C, with several buffer changes. Glycerol was added to the sample for a final concentration

of 10% (v/v) glycerol. The viral concentration was determined by a spectrophotometric reading at A_{260} with an optical density (O.D.) unit of 1 equivalent to 10^{10} PFU/ml (181). Purified AdV were stored at -80°C until use.

4.2.3.4 Bone Marrow (BM)-derived DC

The preparation of bone marrow -derived DCs (BM-DCs) was previously described (182). Briefly, BM cells were collected from the femora and tibiae of naïve C57BL/6 mice. The BM cells were depleted of red blood cells with 0.84% Tris-ammonium chloride and plated in DC culture medium [DMEM with 10% FBS, GM-CSF (20 ng/ml; R&D System, Minneapolis, MN) and IL-4 (20 ng/ml; R&D System, Minneapolis, MN)]. On day 3, the non-adherent granulocytes, T cells and B cells were gently removed, and fresh media was added. Two days later, the loosely adherent proliferating DC aggregates were dislodged and re-plated. On day 6 of culture, non-adherent DC cells were harvested for phenotypic analysis, OVA pulsing *in vitro* or AdV transductions.

4.2.3.5 DC Transduction with AdV

Six-day cultured DCs were incubated with $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} at a multiplicity of infection (MOI) of 150. After viral adsorption for 1 hour at 37°C in DMEM in 6-well culture plates, the DC culture medium was replaced with DMEM containing 10% FBS and these cells were incubated for another 24 h at 37°C. DCs infected with $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} were termed $DC_{AdVIL\text{-}6}$ and $DC_{AdVpLpA}$, respectively. The infected cells were harvested for phenotypic analysis by flow cytometry or examination of IL-6 and the control housekeeping gene GAPDH expression

using RT-PCR (as outlined in Section 4.2.1.1 and Section 4.2.1.2). Moreover, the supernatants of $DC_{AdVIL\text{-}6}$ and $DC_{AdVpLpA}$ were collected to assay the secretion of IL-6 using the IL-6 Enzyme linked immunosorbent assay (ELISA) Kit (BD Bioscience, Mississauga, ON, Canada).

4.2.3.6 Preparation of DC_{OVA}, IL-6 Transgene Engineered DC_{OVA} ($DC_{OVA/AdVIL\text{-}6}$) and pLpA Transgene Engineered DC_{OVA} ($DC_{OVA/AdVpLpA}$)

Non-adherent, 6 day cultured DCs were harvested (as outlined in Section 4.2.3.4), and then pulsed with 0.4 mg/ml OVA overnight at 37°C in serum-free AIM-V medium (Invitrogen, Burlington, ON, Canada). The following morning, these cells were harvested, washed extensively, and referred to as DC_{OVA}. To produce IL-6 transgene engineered DC_{OVA} ($DC_{OVA/AdVIL\text{-}6}$) and pLpA transgene engineered DC_{OVA} ($DC_{OVA/AdVpLpA}$), we incubated these DCs with $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} at a multiplicity of infection (MOI) of 150 for 3 hours at 37°C in AIM-V medium in 6-well culture plates. After viral adsorption, DCs were pulsed with 0.4 mg/ml OVA and AdV overnight at 37°C in the AIM-V medium. These DCs infected with $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} and pulsed with OVA were termed $DC_{OVA/AdVIL\text{-}6}$ and $DC_{OVA/AdVpLpA}$, respectively.

4.2.3.7 Preparation of OVA-specific $AdV_{IL\text{-}6}$ or AdV_{pLpA}-engineered DC-activated $CD8^+$ T Cells

Spleens were removed from OVA-specific TCR transgenic OT I mice and mechanically disrupted to obtain a single-cell suspension. The erythrocytes were lysed using 0.84% ammonium chloride. The naive $CD8^+$ T cells were enriched by passage through nylon

wool columns (C&A Scientific, Manassas, VA), and then further fractionated by negative selection using anti-mouse CD4 (L3T4) paramagnetic beads (Invitrogen, Burlington, ON, Canada). To generate OVA-specific $AdV_{IL\text{-}6}$ or AdV_{pLpA}-engineered DC-activated $CD8^+$ T cells , these naive $CD8^+$ T cells (2×10^5 cells/ml) were stimulated for 72 h with irradiated (4,000 rads) $DC_{OVA/IL\text{-}6}$ or $DC_{OVA/pLpA}$ (1×10^5 cells/ml), respectively, in the presence of IL-2 (10 U/ml). The OVA-specific $AdV_{IL\text{-}6}$ or AdV_{pLpA}-engineered DC-activated $CD8^+$ T cells were referred to as T_A or T_B, respectively.

4.2.4 Immunology Methods

4.2.4.1 Enzyme linked immunosorbent assay (ELISA)

To confirm IL-6 expression in the IL-6 transgene-engineered DCs, an ELISA was performed using a Mouse IL-6 ELISA Set (BD Bioscience, Mississauga, ON, Canada) in accordance with the manufacturer's protocol. Briefly, a 96-well plate was coated with IL-6 capture antibody diluted in coating buffer (PH 9.5) for incubation overnight at 4 °C. The following morning, 96-well plate was washed three times with PBS with 0.05% Tween-20 (Bio-Rad, Mississauga, ON, Canada) and blocked with 3% bovine serum albumin (BSA) (w/v) in PBS for 1 hour at room temperature. The plate was washed twice with PBS with 0.05% (v/v) Tween-20 and standards, along with the culture supernatant of $DC_{AdVIL\text{-}6}$ and $DC_{AdVpLpA}$, as described in Section 4.2.3.5, diluted at some appropriate ratio, were added. The plate was incubated for 2 hour at room temperature. Following this, the plate was washed 5 times with PBS with 0.05% (v/v) Tween-20 and incubated with a 1:1 mixture of Biotin conjugated anti-mouse IL-6 antibody and horseradish

peroxidase (HRP) conjugated-streptavidin for 1 hour followed by additional washes. Next, 3,3',5,5' tetramethylbenzidine (TMB) substrate (TMB Substrate Kit; BD Bioscience, Mississauga, ON, Canada) was added to each well and incubated for 30 min followed by the addition of 2N H_2SO_4 (BDH Inc., Toronto, ON, Canada) to stop the reaction. Developed plates were read on a Bio-Rad microplate reader at a wavelength of 450 nm. Values of samples are found out by plotting OD values against standard curve.

4.2.4.2 Phenotypic Characterization of Engineered DC and the Engineered DC-activated $CD8^+$ T Cells

For phenotypic analysis, DC, $DC_{AdVIL\text{-}6}$ and $DC_{AdVpLpA}$ were stained for 1 h on ice with the biotin-conjugated anti-mouse antibodies (2 mg/ml) specific for major histocompatibility complex (MHC) class Ⅱ (I_a^b), CD40, CD54 or CD80. The cells were then washed three times with PBS, prior to incubation for an additional 1 hour on ice with FITC-conjugated streptavidin. After another three washes with PBS, the cells were analyzed by flow cytometry using an Epics XL flow cytometer (Beckman-Coulter, Mississauga, ON, Canada). Isotype-matched mAbs were used as controls.

The method of analyzing the phenotypic characters of the activated T cells is similar as that of DCs. Briefly, the engineered DC-activated $CD8^+$ T cells, including T_A and T_B, were stained with biotin-conjugated anti-mouse antibodies specific for CD8, CD25 and CD69 followed with FITC-conjugated streptavidin or PE-conjugated anti-mouse antibodies specific for CD62 ligand (CD62L) and Fas ligand (FasL), and then analyzed by flow cytometry. Isotype-matched mAbs were used as controls. To examine the

intracellular expression of cytokines, the above activated T cells were processed using Cytofix/CytoPerm Plus with GolgiPlug kit (BD Bioscience, Mississauga, ON, Canada), and stained with PE-conjugated anti-mouse perforin antibody, according to the manufacturer's protocol (183). 250ul of fixative/permeabilization buffer was added to the engineered DC-activated $CD8^+$ T cells and incubated for 20 min on ice. After being washed three times wtih BD Perm/Wash buffer, the fixed/permeabilized cells were stained with PE-conjugated anti-mouse perforin antibody for 1 hour on ice in the dark, and washed three times with BD Perm/Wash buffer. These cells were then analyzed by flow cytometry. Isotype-matched mAbs were used as controls.

4.2.4.3 Tetramer Staining Assay

The $DC_{OVA/AdVIL\text{-}6}$ or $DC_{OVA/AdVpLpA}$ were injected intravenous (i.v.) into naïve C57BL/6 mice (six mouse/group, 1×10^6 cells /mouse). PBS was used as a control. Six days after immunization, 100μl of blood was taken from the tail vein and the blood samples were stained with PE-labeled H-$2K^b$/OVA $_{257\text{-}264}$ tetramer (Beckman-Coulter, Mississauga, ON, Canada) and FITC-labeled anti CD8 antibody for 30 min at room temperature in the dark. Lysis/fixative buffer (Beckman-Coulter, Mississauga, ON, Canada) was used to lyse the erythrocytes. The cells were washed three times with PBS and analyzed by flow cytometry using an Epics XL flow cytometer (Beckman-Coulter, Mississauga, ON, Canada).

4.2.4.4 *In vivo* Cytotoxicity Assay

The *In vivo* cytotoxicity assay was performed as described previously (182). To generate differentially labeled target cells, Splenocytes derived from naïve C57BL/6 mice were incubated with high (3.0μM, $CFSE^{high}$) or low (0.6μM, $CFSE^{low}$) concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE). $CFSE^{high}$ cells were pulsed with the OVA I peptide, SINFEKL, and washed extensively to remove free peptide. However, the $CFSE^{low}$ cells were not pulsed to become the internal controls. $CFSE^{high}$ and $CFSE^{low}$ were co-injected i.v. at a ratio of 1:1 into the above immunized mice described in Section 4.2.4.3, six days after the final immunization. Sixteen hours after injection, spleens were removed from the immunized mice to analyze residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in recipients' spleens by flow cytometry.

4.2.4.5 T Cell Survival

Naïve C57BL/6 mice were injected i.v. with engineered DC-activated $CD8^+$ T cells (T_A or T_B, 5×10^6 cells /mouse). Six, twelve and thirty days after adoptive transfer, blood was taken from the tail vein, and analyzed with the H-$2K^b$/OVA $_{257\text{-}264}$ tetramer assay (as outlined in Section 4.2.4.3) to detect the presence of OVA-specific $CD8^+$ T cells in the mouse peripheral blood.

4.2.5 Animal Studies

4.2.5.1 DC Vaccine

For evaluation of tumor prevention, naïve C57BL/6 mice were vaccinated i.v. with 1×10^6 engineered $DC_{OVA/AdVIL\text{-}6}$, $DC_{OVA/AdVpLpA}$ or the control PBS, respectively. On day 8, the mice (8 mice per group) were challenged by i.v. injection with two different doses of OVA transgene-expressing BL6-10_{OVA} tumor cells, 0.5×10^6 and 1×10^6, respectively. Four weeks after tumor cell challenge, these mice were sacrificed and the number of lung metastatic tumor colonies was counted. The lung tissues of these mice were further analyzed by histological examination.

4.2.5.2 Adoptive OVA-specific $AdV_{IL\text{-}6}$ or AdV_{pLpA}-engineered DC-activated $CD8^+$ T Cell Immunotherapy Model

Naïve C57BL/6 mice (8 per group) were subcutaneously (s.c.) inoculated with 1×10^6 OVA transgene-expressing EG7 tumor cells in their right thighs. After 10 to 11 days, when the tumors reached around 8mm in diameter, each mouse received an i.v. injection of 2×10^6 $AdV_{IL\text{-}6}$ or AdV_{pLpA}-engineered DC-activated $CD8^+$ T cells or the control PBS. By measuring two perpendicular tumor diameters using a caliper, tumor growth or regression were monitored daily. For humanitarian reasons, all mice showing severe distress or with tumors that achieved a size of 1.7 cm in diameter were killed. Animals were monitored for a total of 70 days. To analyze the anti-tumor immune memory, 8 weeks after tumor regression, mice showing a decrease in tumor size were re-challenged

with a s.c. injection of 1×10^6 EG7 tumor cells. The tumor growth was monitored as above. Naïve C57BL/6 mice were used as controls.

4.2.6 Histological Examination

The lung tissues were removed from the immunized mice four weeks after tumor cell challenge (as referred in Section 4.2.5.1). The tumor samples were fixed in 10% formaldehyde, embedded in paraffin and sliced into sections of 6–7μm thickness. These sections were stained with hematoxylin-eosin in accordance with the standard procedures.

4.2.7 Statistical Analysis

To compare mouse survival and tumor development between groups, Log-rank tests were performed. The significance of differences was determined between groups by Student's t-test using Stat View SE Software (Abacus Concepts, Berkeley, CA). The difference was considered statistically significant at P values less than 0.05 ($P<0.05$).

Chapter 5

RESULTS

5.1 Part A- IL-6 Engineered Dendritic cell (DC) Vaccine

5.1.1 Construction of AdV

To construct the early transcribed regions (E)-1/E3-deleted replication-deficient adenovirus (AdV) vectors, we used the AdEasy system under the regulation of the cytomegalovirus (CMV) early/immediate promoter/enhancer, which is shown in figure 5.1A. Since DCs express low levels of 'Coxsackie virus and Adenovirus receptor' (CAR) and high levels of surface integrins (161-163), a arginine-guanine-aspartate (RGD) motif, which can bind to the integrins and stimulate AdV internalization (160, 184), was added to the HI loop of the AdV fiber. It is reported that this method can improve gene transfer to dendritic cells (DCs) (164-166). As shown in figure 5.1B, $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} are the RGD-modified AdV with IL-6 and no transgene insert, respectively. Moreover, $AdV_{IL\text{-}6}$ infected DCs will now be referred to as $DC_{AdVIL\text{-}6}$, and AdV_{pLpA} infected DCs will be referred to as $DC_{AdVpLpA}$.

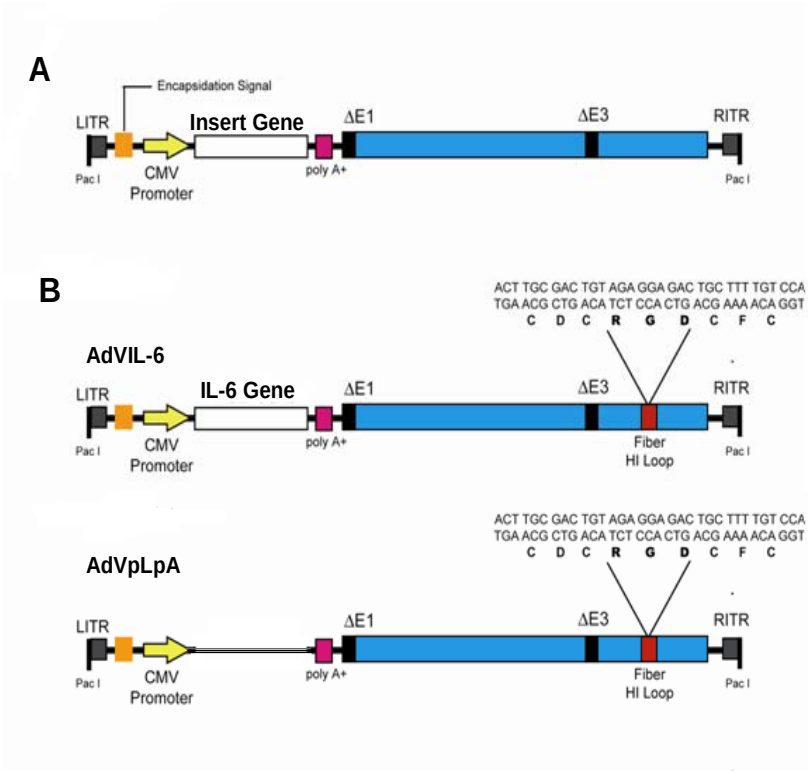

Figure 5. 1: Constructed Adenoviral vectors.

(**A**)The E1/E3-deleted replication-deficient AdV vectors are under the regulation of the CMV early/immediate promoter/enhancer. (**B**) The RGD-modified AdV was fiber-modified form of AdV containing the RGD peptide in the HI loop of the fiber knob. $AdV_{IL\text{-}6}$ and the control AdV_{pLpA} are the RGD-modified AdV with the IL-6 gene and no transgene insert, respectively. ITR, inverted terminal repeat.

5.1.2 IL-6 Expression in $DC_{AdVIL-6}$

To examine IL-6 expression, RNA extracted from infected $DC_{AdVIL-6}$ and $DC_{AdVpLpA}$ was subjected to RT-PCR analysis. As shown in Figure 5.2A, the expression levels of a house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were similar, indicating that similar amounts of RNA from $DC_{AdVIL-6}$ and $DC_{AdVpLpA}$ were loaded. Moreover, a significant amount of IL-6 expression was found in the AdV_{IL-6}-infected $DC_{AdVIL-6}$, but not in the control adenovirus-infected $DC_{AdVpLpA}$, suggesting the difference seen in the IL-6 expression levels resulted from AdV_{IL-6} infection. To further analyze the quantity of IL-6 expression, we conducted an IL-6 detection via Mouse IL-6 ELISA Kit (BD Bioscience, Mississauga, ON, Canada). As shown in figure 5.2B, the secretion of IL-6 was estimated to be around 1.8μg/ml/10^6/24h, 0.6μg/ml/10^6/24h and 0.02μg/ml/10^6/24h for $DC_{AdVIL-6}$, $DC_{AdVpLpA}$ and untreated DCs, respectively. This indicates that AdV infection significantly increases the IL-6 secretion from nominal level to microgram level and AdV_{IL-6} infected DCs express a higher level of the IL-6 due to the transgene IL-6 expression.

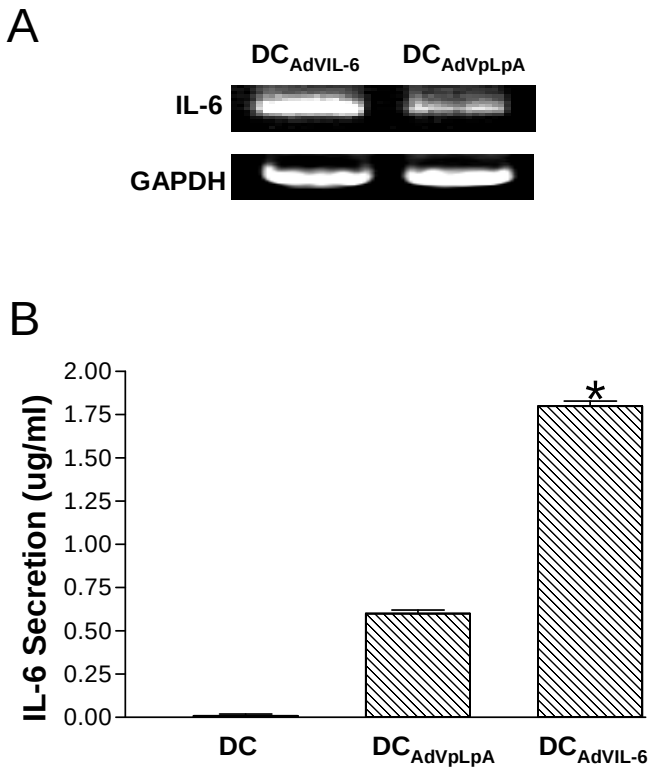

Figure 5. 2: Expression of cytokine IL-6.

(A) RNA was obtained from AdV_{IL-6} infected $DC_{AdVIL-6}$ and the control AdV_{pLpA} infected $DC_{AdVpLpA}$ cells for examination of IL-6 expression. The first-strand cDNA was synthesized from RNA using reverse transcriptase, and the PCRs were conducted using sets of primers for IL-6 and the control 'house-keeping' gene GAPDH, respectively. After 30 cycles, 10µl of the reaction was run on a 1.5% agarose gel. **(B)** The supernatants of $DC_{AdVIL-6,}$ $DC_{AdVpLpA}$ and untreated DCs were measured for expression of IL-6, respectively, using an IL-6 ELISA Kit.

5.1.3 AdV Enhances the Maturation of DCs

To assess the phenotypic changes on $AdV_{IL\text{-}6}$-infected DCs, $DC_{AdVIL\text{-}6}$, $DC_{AdVpLpA}$ as well as untreated DCs were subjected to phenotypic analysis using flow cytometry. As shown in figure 5.3, both $AdV_{IL\text{-}6}$-infected DCs ($DC_{AdVIL\text{-}6}$) and control AdV_{pLpA}-infected DCs ($DC_{AdVpLpA}$) showed a mild to moderate up-regulation of MHC class Ⅱ (I_a^b), CD40, CD54 and CD80 expression, compared to untreated DCs. Since up-regulation of cellular expression of these immunological molecules is related to DC maturation, our results indicate that AdV-infected DCs become a more mature form of DCs. However, the IL-6 transgene itself does not affect DC maturation

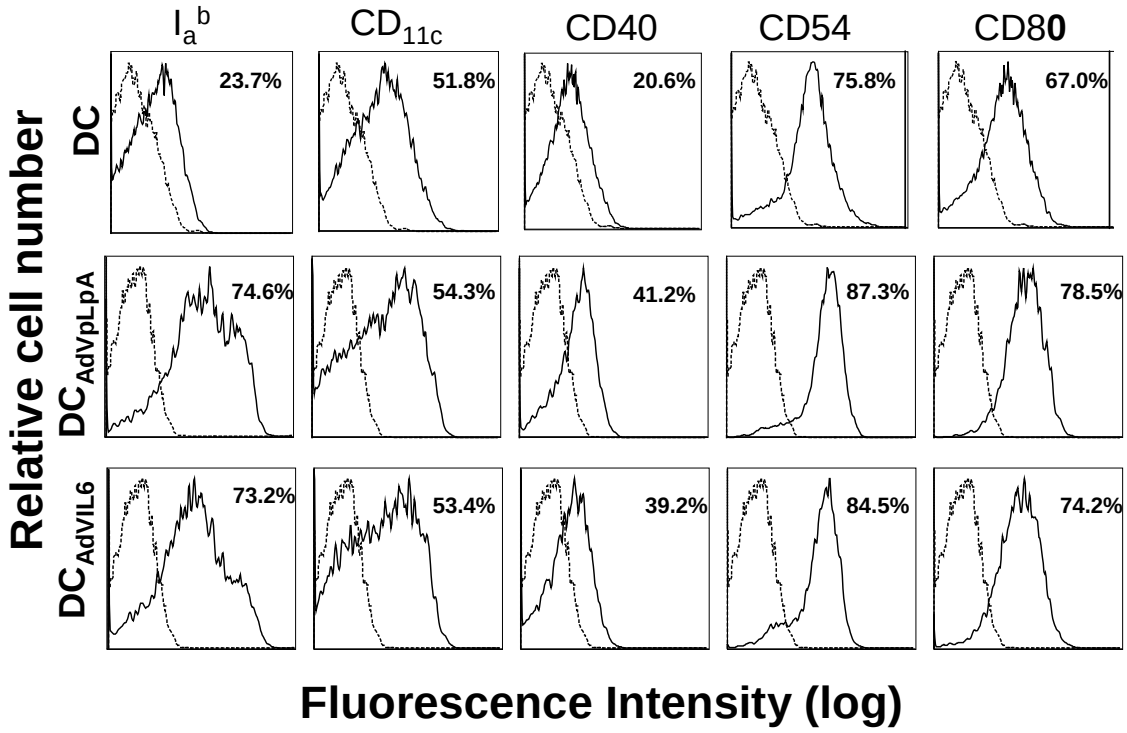

Figure 5. 3: Adenovirus enhances the maturation of DCs.

The phenotypic changes of DCs were compared by flow cytometry. Un-infected DCs, AdV_{pLpA}-infected DCs ($DC_{AdVpLpA}$) and $AdV_{IL\text{-}6}$-infected DCs ($DC_{AdVIL\text{-}6}$) were harvested and analyzed for surface expression of MHC class Ⅱ (I_a^b), CD_{11c}, CD40, CD54 and CD80 molecules using the mouse anti- MHC class Ⅱ (I_a^b), CD_{11c} , CD40, CD54 and CD80 antibodies and the FITC-labeled goat anti-mouse antibody (solid lines). Isotype-matched monoclonal antibodies (dashed lines) were used as controls. The value in each panel represents the percentage of positive cells based on the isotype control. One of two representative experiments is shown.

5.1.4 The $DC_{OVA/AdVIL-6}$ Vaccine Stimulates a Higher Percentage of OVA-specific $CD8^+$ T Cells than the Control $DC_{OVA/AdVpLpA}$ Vaccine

Naïve C57BL/6 mice were immunized with $DC_{OVA/AdVIL-6}$ or $DC_{OVA/AdVpLpA}$. Six days later, the amount of OVA-specific $CD8^+$ T cells in the peripheral blood was measured using PE-labeled H-$2K^b$/$OVA_{257-264}$ tetramer staining and FITC-CD8 staining. As illustrated in figure 5.4, the percentage of double positive (PE-$tetramer^+$ and FITC-$CD8^+$) cells in the total $CD8^+$ population is significantly higher in the $DC_{OVA/AdVIL-6}$ immunized mice (0.6%) compared to the $DC_{OVA/AdVpLpA}$ immunized mice (0.14%) (Student t-test, P-value< 0.05), with both immunized groups showing a significant difference compared to the control PBS immunized mice (Student t-test, $P<0.05$). This indicates that $DC_{OVA/AdVIL-6}$ immunization stimulates a stronger OVA-specific $CD8^+$ T cell response, compared to $DC_{OVA/AdVpLpA}$ immunization.

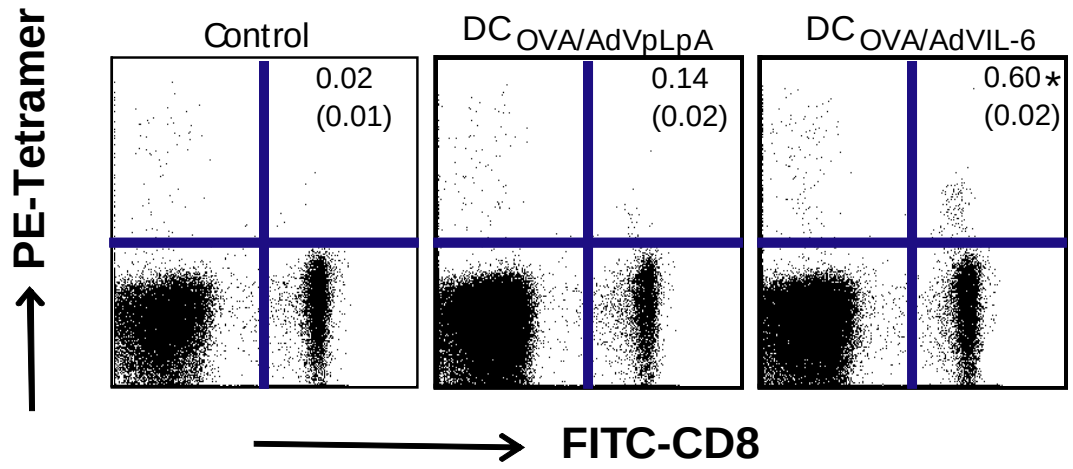

Figure 5. 4: $DC_{OVA/AdVIL-6}$ vaccine stimulates a higher percentage of OVA-specific $CD8^+$ T cells response than $DC_{OVA/AdVpLpA}$.

The $DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$ were i.v. injected into naïve C57BL/6 mice (6 mice per group, 1×10^6 cells per mouse). Mouse tail blood cells were stained with PE-H-$2K^b$/OVAI tetramer (PE-tetramer) and FITC-anti-CD8 antibody (FITC-CD8), and analyzed by flow cytometry at day six after immunization. The value in each panel represents the percentage of PE tetramer- positive $CD8^+$ T cells versus the total peripheral $CD8^+$ T-cell population. The value in parentheses represents the standard deviation (SD). A significant difference exists for both the $DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$ immunized groups compared to the control PBS (Student t-test, $P<0.05$). More importantly there was a significant difference between the $DC_{OVA/AdVIL-6}$ immunized and $DC_{OVA/AdVpLpA}$ immunized groups ($p<0.05$), indicated by *. One of two representative experiments is shown.

5.1.5 The $DC_{OVA/AdVIL-6}$ Vaccine was More Efficient in Stimulating $CD8^+$ T Cell Differentiation into Effector $CD8^+$ CTL than the Control $DC_{OVA/AdVpLpA}$ Vaccine.

To analyze the ability of $DC_{OVA/AdVIL-6}$ to induce the differentiation of naïve $CD8^+$ T cell into effector CTL, an *in vivo* cytotoxicity assay was performed. We adoptively i.v. transferred OVAI peptide pulsed splenocytes, strongly labeled with CFSE ($CFSE^{high}$), as well as weakly labeled with CFSE ($CFSE^{low}$, non-peptide pulsed), into recipient mice, which had been vaccinated with $DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$, respectively. Flow cytometric analysis was performed to examine the ability of activated T cells to induce specific killing of labeled splenocytes (target cells). In Figure 5.5, because cell killing was specifically targeted towards OVA labeled cells, levels of $CFSE^{low}$ cells remained unaffected and the level of $CFSE^{high}$ cells remaining in the spleen was reduced accordingly. Residual $CFSE^{high}$ cells remained unchanged in the spleen of the control mice and there was substantial loss of the $CFSE^{high}$ (OVAI peptide pulsed) cells in immunized mice. Among them, the mice immunized with $DC_{OVA/AdVpLpA}$ had a decrease of 46% $CFSE^{high}$ (OVAI peptide pulsed) cells, whereas the mice immunized with $DC_{OVA/AdVIL-6}$ had a greater degree of loss (93.3%). This result indicates that $DC_{OVA/AdVIL-6}$ cells were more efficient in stimulating $CD8^+$ T cell differentiation into effector $CD8^+$ CTL than $DC_{OVA/AdVpLpA}$.

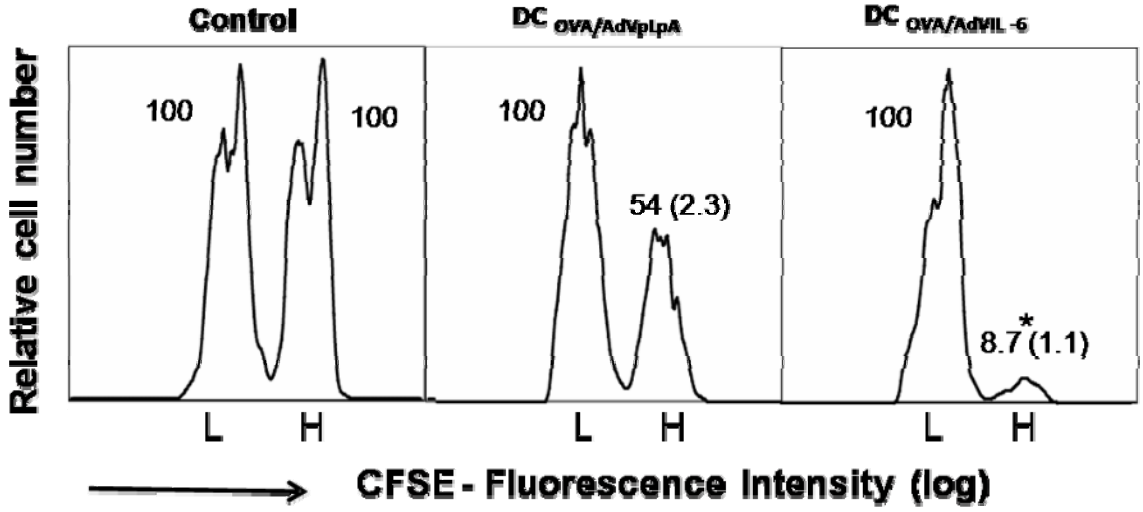

Figure 5. 5: $DC_{OVA/AdVIL-6}$ vaccine stimulates $CD8^+$T cell differentiation more efficiently into effector $CD8^+$ CTL than $DC_{AdVpLpA}$.

In the *in vivo* cytotoxicity assay, C57BL/6 mice were immunized i.v. with $DC_{OVA/AdVIL-6}$, $DC_{OVA/AdVpLpA}$ or PBS. Six days after immunization, the mice were co-injected i.v. at a 1:1 ratio of splenocytes labeled with high (3.0 μM, $CFSE^{high}$) and low (0.6 μM, $CFSE^{low}$) concentrations of CFSE and pulsed with OVA I and non-peptide, respectively. Sixteen hours after target cell delivery, the residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the spleens of the recipients were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ cells versus $CFSE^{low}$ cells remaining in the spleens. The value in parentheses represents the SD. *, $P < 0.05$, versus cohorts of $DC_{OVA/AdVpLpA}$ cells (Student's *t*-test). One representative experiment of three is shown. L, Low; H, high.

5.1.6 The $DC_{OVA/AdVIL-6}$ Vaccine Induces More Efficient Protection against Tumor Challenge than the Control $DC_{OVA/AdVpLpA}$ Vaccine

To study whether $DC_{OVA/AdVIL-6}$ are also capable of inducing enhanced antitumor immunity *in vivo*, we vaccinated mice with $DC_{OVA/AdVIL-6}$, $DC_{OVA/AdVpLpA}$ and control PBS. Eight days later, both low (0.5×10^6 cells, Experiment I) and high (1×10^6 cells, Experiment II) doses of BL6-10_{OVA} tumor cells were used to challenge the immunized C57BL/6 mice. Four weeks after tumor cell challenge, these mice were sacrificed and the number of lung metastatic tumor colonies was counted. The lung tissues from these sacrificed mice were removed, fixed in 10% formaldehyde and embedded in paraffin for histological analysis. Sections of 6–7μm thickness were stained with hematoxylin-eosin according to standard procedures. As shown in table 5.1, after challenge with a low dose of BL6-10_{OVA} tumour cells, vaccination with either $DC_{OVA/AdVIL-6}$ or $DC_{OVA/AdVpLpA}$ was sufficient to protect 100% of the mice from tumour growth (8/8 protected). However, when mice were challenged with a high dose of tumour cells, $DC_{OVA/AdVpLpA}$ immunization was only able to protect 50% of the mice (4/8 protected) while $DC_{OVA/AdVIL-6}$ immunization was still able to protect 100% of the mice from tumor growth (8/8 protected). Therefore, vaccination with any of the OVA-pulsed AdV-infected DC populations ($DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$) is sufficient to induce protection against a challenge of low dose of tumour cells. A distinct effect of immune protection derived from differential DC vaccination was only apparent when a higher dose of tumor cells are used. As shown in figure 5.6, lung metastases were in the section of lung tissue from the mouse immunized with $DC_{OVA/AdVpLpA}$, but not in lung tissue from the mouse

immunized with $DC_{OVA/AdVIL-6}$. These data indicate that vaccination using $DC_{OVA/AdVIL-6}$ cells is able to induce a stronger antitumor immunity than similar vaccination using $DC_{OVA/AdVpLpA}$.

Table 5. 1: The $DC_{OVA/AdVIL-6}$ vaccine induces more efficient protection against tumor challenge than the control $DC_{OVA/AdVpLpA}$ vaccine

T cell transfer	Tumor cell challenge	Tumor bearing mice (%)	Median number of lung tumor colonies
Exp. I[A]			
$DC_{OVA/AdVpLpA}$	BL6-10_{OVA}	0/8 (0)	0
$DC_{OVA/AdVIL-6}$	BL6-10_{OVA}	0/8 (0)	0
PBS	BL6-10_{OVA}	8/8 (100)	>100
Exp. II			
$DC_{OVA/AdVpLpA}$	BL6-10_{OVA}	4/8 (50)	49±23
$DC_{OVA/AdVIL-6}$	BL6-10_{OVA}	0/8 (0)	0
PBS	BL6-10_{OVA}	8/8 (100)	>100

A. Naïve C57BL/6 mice were immunized i.v. with engineered $DC_{OVA/AdVIL-6}$, $DC_{OVA/AdVpLpA}$ and the control PBS, respectively. In experiment I, each mouse was injected i.v. with 0.5x 10^6 OVA transgene-expressing BL6-10_{OVA} tumor cells eight days after immunization. In experiment II, each mouse was injected i.v. with 1x 10^6 BL6-10_{OVA} tumor cells eight days after immunization. These mice were sacrificed 4 weeks after tumor cell challenge and the number of lung metastatic tumor colonies was counted. One representative experiment of three is shown.

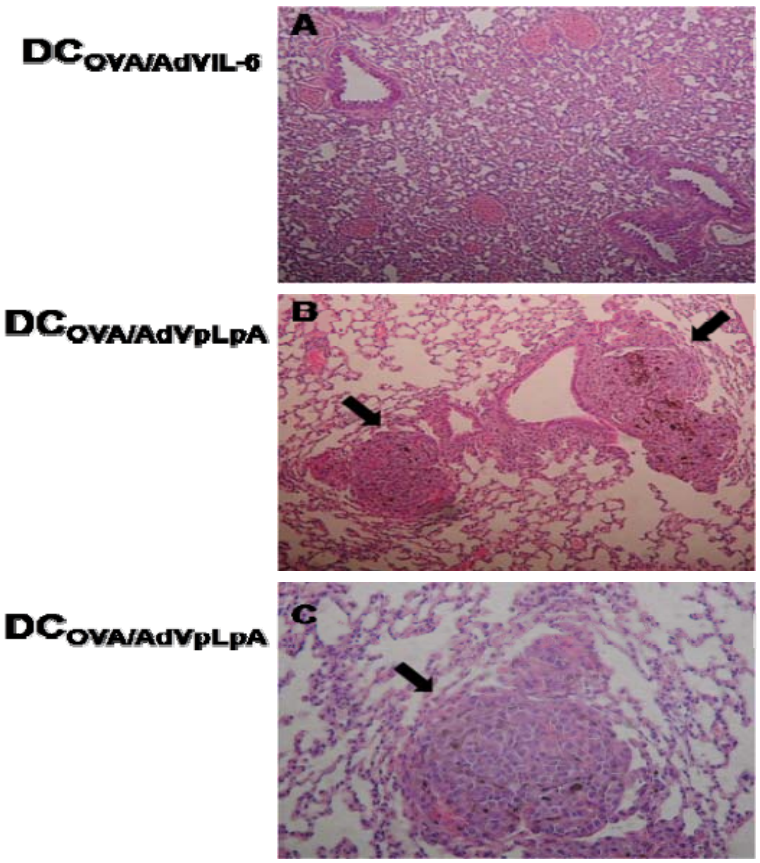

Figure 5. 6: Histological photomicrographs of lung tissue.

Naïve C57BL/6 mice were immunized i.v. with engineered $DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$, respectively. Eight days after immunization, each mouse was injected i.v. with 1x 10^6 $BL6\text{-}10_{OVA}$ tumor cells. These mice were sacrificed 4 weeks after tumor cell challenge. (**A**) Section of the lung tissue from the mouse immunized with $DC_{OVA/AdVIL-6}$ shows no lung metastasis. (**B**) and (**C**) Sections of the lung tissue from the mouse immunized with $DC_{OVA/AdVpLpA}$ show lung metastatic tumor (arrows). All figures A to C stained by hematoxylin and eosin. Magnifications were x10 for A and B, and x20 for C.

5.2 Part B- Immune Mechanism underlying the Result of IL-6 Engineered-DC Vaccine

5.2.1 $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T Cells Display Higher Level of CD62L, FasL and Perforin than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T Cells

To produce the active $DC_{OVA/AdVIL-6}$-activated and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells, naïve $CD8^+$ T cells isolated from OT I mice were incubated with irradiated $DC_{OVA/AdVIL-6}$ and $DC_{OVA/AdVpLpA}$ for 72 h, respectively. As shown in Figure 5.7, both of these $CD8^+$ T cells displayed the $CD8^+$ T cell marker (CD8) as well as CD25 and CD69, indicating that they are highly activated. However, $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells display higher level of CD62L than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells, indicating that $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells have a stronger migration activity and a tendency to become memory T cells. Moreover, compared to $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells, FasL and perforin, the markers of cytotoxicity, were also highly up-regulated in $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells. This result indicates that $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells have an increased cytotoxic ability.

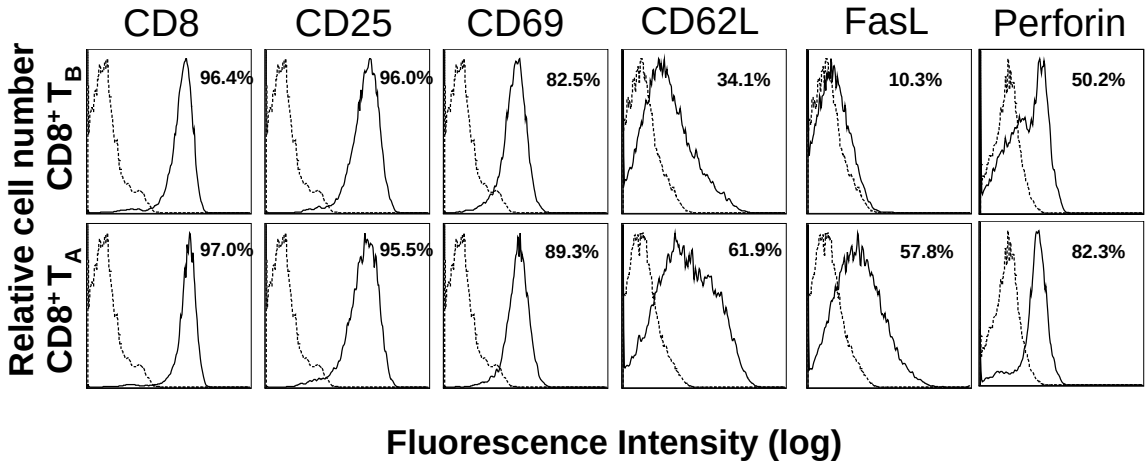

Figure 5. 7: Phenotypic analysis of $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B).

Naïve $CD8^+$ T cells were separated from spleen cells of OT I mice, and then activated *in vitro* by incubation with irradiated $DC_{OVA/AdVIL\text{-}6}$ and $DC_{OVA/AdVpLpA}$ for 72 h, respectively. The activated $CD8^+$ T_A cells and $CD8^+$ T_B cells were stained using a panel of FITC-labelled mAbs (solid lines) for analysis of CD8, CD25, CD69, CD62L, FasL and Perforin, or isotype-matched mAbs (dotted lines). The value in each panel represents the percentage of positive cells based on the isotype control. Data are representative of two experiments with three mice per group.

5.2.2 $DC_{OVA/AdVIL-6}$-activated $CD8^+$ CTL have Prolonged $CD8^+$ T Cell Survival

A successful DC-based vaccine is critically dependent upon the duration of *in vivo* persistence of the activated $CD8^+$ T cells. To analyze the survival of $DC_{OVA/AdVIL-6}$-activated $CD8^+$ CTL cells *in vivo*, we performed a kinetic study. In the kinetic study, we measured the percentage of OVA-specific $CD8^+$ T cells in the peripheral blood of naïve C57BL/6 mice with i.v. transfer of $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) using PE-labeled H-$2K^b$/$OVA_{257–264}$ tetramer staining and FITC-CD8 staining at different time points after adoptive transfer. As illustrated in Figure 5.8, the number of total OVA-specific $CD8^+$ T cells detected in the peripheral blood of the mice with $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) transfer accounted for 2.54% and 2.37% of the total $CD8^+$ T-cell population, respectively, at day 6 after adoptive transfer. The numbers then gradually dropped to 1.45% and 1.00% at day 12, and 1.23% and 0.38% at day 30 after adoptive transfer. The number of $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) dropped much faster than that of $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A), indicating that the $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) have prolonged survival *in vivo*, compared with the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B).

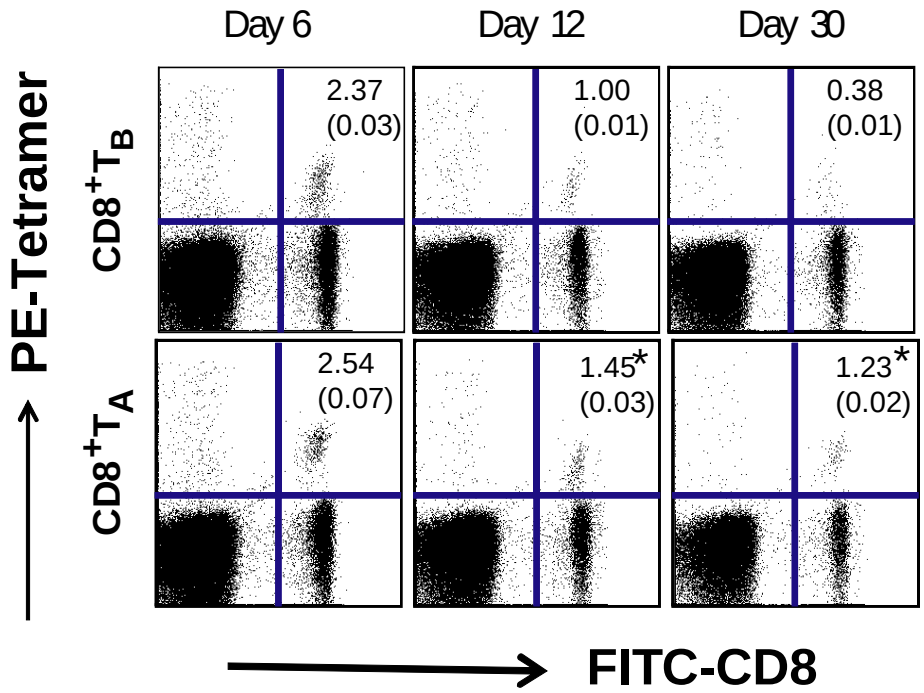

Figure 5. 8: $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) have prolonged $CD8^+$ T cell survival.

The $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) and the $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) were injected i.v. into naïve C57BL/6 mice (6 mice per group). Mouse tail blood cells were stained with PE-H-$2K^b$/OVAI tetramer (PE-tetramer), FITC-anti-CD8 antibody (FITC-CD8) and analyzed by flow cytometry at the indicated time points after adoptive transfer. The value in each panel represents the percentage of PE tetramer-positive $CD8^+$ T cells versus the total peripheral $CD8^+$ T-cell population. The value in parentheses represents the standard deviation (SD). *$P<0.05$ versus cohorts of AdV_{pLpA}-engineered DC-activated $CD8^+$ T cells (T_B) (Student's t-test). One representative experiment of three is shown.

5.2.3 $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ CTL have Stronger Antitumor Immunity than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ CTL and IL-6 Transfection can Enhance $CD8^+$ Memory T Cell Development.

In clinical practice, most candidates for cancer therapy are patients with existing tumor burdens. In modeling the clinical case precisely, we must direct our questions of therapeutic efficacy to the eradication of established tumor. To target this, we tested the effects of $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) using a 10-day established EG7 tumor model system. Naïve C57BL6 Mice received s.c. injections of 1×10^6 EG7 tumor cells in their right thighs. At 10-12 days post-inoculation, these tumors were around 8 mm in diameter and had well-developed vasculature. We then injected i.v. 2×10^6 $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) or $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B), respectively. Control mice were given i.v. injections of PBS. Tumor growth within the mice and their mortality rates were then monitored daily for up to 70 days. For humanitarian reasons, all mice with tumors that achieved a size of 1.7 cm in diameter were sacrificed. Figure 5.9 shows that in the control group, the tumors grew very aggressively and all of the mice in this group died within 10 days of the treatment onset; in the $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B) treated group, the tumors grew more slowly than control group but all of the mice in this group died within 20 days of the treatment onset. By contrast, all of the mice with tumors that had been treated with the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) were cured. These results suggest that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ CTL have stronger antitumor immunity than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ CTL.

To observe the antitumor immune memory, mice cured of their tumors via $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) were again challenged with 1×10^6 EG7 tumor cells 8 weeks subsequent to the tumor regression. The mice in control group were naïve C57BL6 mice. The tumor growth was then monitored as above. As shown in figure 5.10, 5 of 8 mice that had treated with $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) did not grow tumors and others (3 of 8) grew small tumors, which disappeared within 20 days of the tumor inoculation. However, tumors within the control mice grew very aggressively and all of the mice in this group died within 20 days of the tumor inoculation. This indicates that $DC_{OVA/AdVIL-6}$-activated $CD8^+$ CTL develop into $CD8^+$ memory T cells more efficiently than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ CTL. Thus, IL-6 secretion would be beneficial to the development of $CD8^+$ memory T cells.

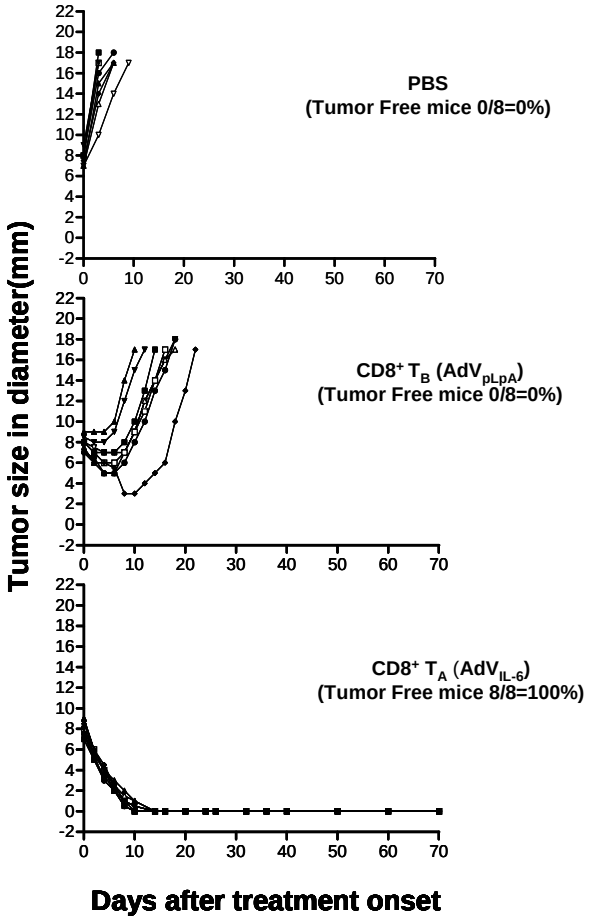

Figure 5. 9: $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A) have stronger antitumor immunity than $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B).

OVA-expressing EG7 tumor cells (1×10^6 cells per mouse) were injected s.c. into the right thighs of C57BL6 mice (8 per group). At ten to eleven days post-inoculation, when the tumors were around 8 mm in diameter, each mouse received an i.v. injection of 2×10^6 $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A), $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells (T_B), or PBS, respectively. Tumor growth or regression was monitored by measuring tumor diameter using a caliper daily for up to 70 days. For humanitarian reasons, all mice that had tumors with 1.7cm in diameter were sacrificed.

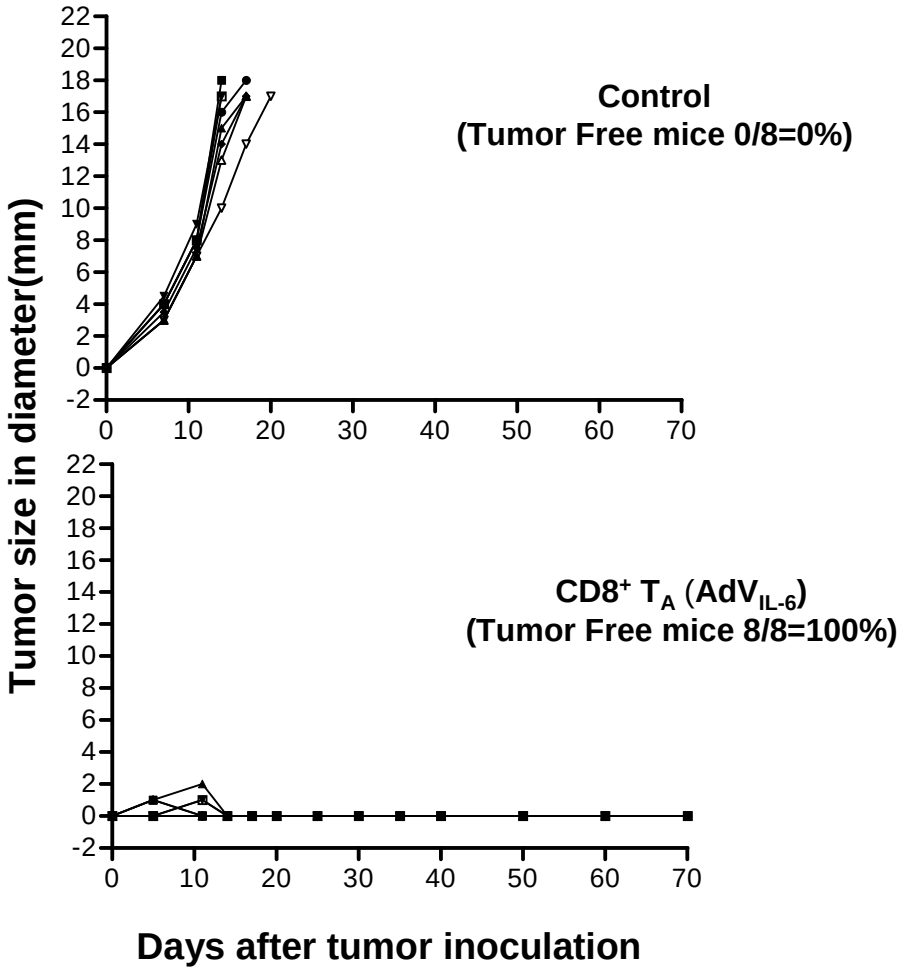

Figure 5. 10: $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) enhance $CD8^+$ memory T cell development.

The mice in the $DC_{OVA/AdVIL-6}$-activated $CD8^+$ T cells (T_A) group were cured of their tumors. These mice were again challenged with $1x10^6$ EG7 tumor cells 8 weeks subsequent to the tumor regression. At the same time, naïve C57BL6 mice, as a control group (8 mice per group), were challenged with EG7 tumor cells. The tumor growth was then monitored as above.

Chapter 6

DISCUSSION

6.1 AdV Enhance the Maturation of DCs

DCs, as professional antigen presenting cells, can uptake antigen efficiently and have high stimulatory activity for T cells (185). In the periphery, the immature DCs capture the antigen. This stimulates maturation process, which allows DCs to migrate to regional lymph nodes to present antigens to T cells and B cells. DCs have two different stages of maturation, immature and mature. Immature DCs are able to capture the antigen efficiently whereas they are poor at presenting the antigen to T cells and B cells. On the contrary, the mature DCs can not efficiently capture the antigen, but have a potent ability to stimulate T cells. Previous reports have shown that immature DCs have low surface expression of MHC class Ⅱ (I_a^b) protein, integrin (CD54) and some costimulatory molecules (CD40 and CD80) (186, 187). However, mature DCs are characterized by high level of expression of these surface markers (185). DCs are required to be in the mature stage to stimulate the immune response and these markers play an important role in the process of stimulating T cells. It has been demonstrated that generation of anti-tumor immunity by DC vaccines is related to the degree of DC maturation (188).

The human type 5 replication-deficient recombinant AdV vectors are commonly used for transgene delivery. Some previous reports have shown that AdV can mature and activate DCs, and can increase the expression of some maturation markers on the surface

of DCs, such as MHC class Ⅱ (I_a^b) protein, integrin (CD54) and some costimulatory molecules (CD40 and CD80) (162, 189-191). Moreover, a recent study showed that the Adenovirus-induced maturation of DCs was linked to activation of a phosphatidylinositol-3-kinase (PI3K)-mediated TNF-α induction pathway (192). Both lipopolysaccharide (LPS) and AdV can activate and mature DCs through an increase in tumor necrosis factor-α (TNF-α) expression. LPS activates DC through Toll-like receptor 4 (TLR-4). TLR-4 signals through MyD88-dependent and MyD88-independent pathway (193, 194), can activate transcription factor, nuclear factor (NF)-κB (195), and then induce the expression of TNF-α. However, unlike the mechanism of LPS-mediated activation and maturation of DCs, the induction of TNF-α expression via the AdV is related to the integrin-mediated PI3K pathway to active NF-κB. During AdV infection, the knob domain located at the end of Ad fiber binds to the CAR on the target cell. After the attachment step, interaction between the RGD modif located on the penton base and the αvβ3 or αvβ5 integrins leads to the internalization of the virus through endocytosis. However, since DCs do not express CAR but high levels of αvβ3 or αvβ5 integrins, the DC infection by AdV was via the binding of the penton base RGD motif of AdV to αvβ3 or αvβ5 integrins of DCs. It was suggested that both DC activation and the TNF-α expression by AdV were also related to the same mechanism (192).

In this study, our data show that the AdV infected $DC_{AdVIL-6}$ and $DC_{AdVpLpA}$ up-regulated the expression of immunologically important molecules (MHC class Ⅱ (I_a^b), CD40, CD54 and CD80), compared with the un-infected DCs, indicating the AdV infected DCs are more mature. Moreover, there are no differences between $DC_{AdVIL-6}$ and

$DC_{AdVpLpA}$ in the expression levels of these immunologically important molecules, indicating the transgene IL-6 expression itself did not affect DC maturation. This is in agreement with the previous literature showing that maturation of DC is dependent only on virus entry but not virus gene or transgene expression (196).

6.2 Engineered DC Vaccine and IL-6 Transgene Engineered DC Vaccine

DCs play a key role in stimulating T cells that are critical for anti-tumor immunity. Based on the role of DCs in immune system, DC-based vaccines have been anticipated as a potential treatment for cancer. DCs are engineered to become potential vaccines against tumors via pulsing with TAA or peptides and delivery of genes encoding tumor-associated antigens into DCs. These antigens can be presented on the surface of DCs with the MHC class I molecule, leading to death of the tumor cells through the induction of CTL-medicated immune responses. As a further strategy to enhance the ability of DC vaccines to induce anti-tumor responses, some genes encoding immunomodulatory proteins such as cytokines and chemokines have been transferred into DCs. As such, the granulocyte macrophage-colony stimulating factor (GM-CSF) –engineered DC can augment the antigen-presenting capacity and the induction of anti-tumor immunity (60, 197). It was reported that DCs expressing IL-12 transgene can promote induction of tumor specific $CD4^+$ T helper (Th) cells and $CD8^+$ CTLs, which enhanced antitumor immunity (65, 198). Recently, some reports suggested that the secondary lymphoid tissue chemokine (SLC) can induce antitumor responses and the i.t. injection of SLC-expressing DCs can inhibit tumor cells in animal models (93, 94, 199). In our lab, we have studied

DC vaccines that were genetically modified to express Fms-like tyrosine kinase 3-ligand (Flt3L), TNF-α or co-stimulatory molecule 40 ligand (CD40L) in the animal models. It has been reported that all of these engineered DC vaccines can stimulate the enhanced specific CTL responses and augment antitumor immunity *in vivo* (67-69).

The cytokine IL-6 exits widely in organ systems, including the immune system, central nervous system, cardiovascular system, hepatic system among others (136). In the immune system, IL-6, secreted by T cells and macrophages, acts as a pro-inflammatory cytokine that can up-regulate adhesion molecules and induce or augment the production of other pro-inflammatory cytokines (200, 201). In addition, IL-6 promotes B cell differentiation and proliferation (202, 203). More recently, it has been reported that IL-6 is a key signal in the development of Th17 cells, but inhibits the formation of the regulatory T (Treg) cells (115). Transforming growth factor (TGF)-β signals can induce naïve $CD4^+$ T cells toward the suppressor Treg cells. The existence of Treg cells in the tumor microenvironment can help tumor cells to escape the lytic activity of activated $CD8^+$ CTL, which limits the therapeutic effect of DC-based vaccines. However, addition of IL-6 to TGF-β can stimulate Th cells to become Th17 cells, not Treg cells (116). Therefore, IL-6 can overcome the Treg-mediated suppression of antigen-specific T cell responses in the tumor microenvironment. This function of IL-6 is similar to TNF-α. Valencia X *et al* have demonstrated that TNF-α can also inhibit the suppressive function of Treg cells by down-regulating FoxP3 expression (79). At the same time, TNF-α infected DCs can also show enhanced antitumor immunity (68). Moreover, it was suggested that IL-6 has anti-apoptotic activity on a wild variety of cells, including the

naïve and activated T cells (204-206). IL-6 inhibits apoptosis of activation-induced cell death of T cells through reduction of the level of T cell receptor (TCR)/CD3-induced apoptosis and expression of Fas/ Fas ligand (FasL), but does not reduce the activation of T cells(153). However, the impact of genetically modifying DCs with the IL-6 gene has not been studied. All of this prompted us to investigate whether enhanced expression of IL-6 is able to increase the antitumor immunity response in a DC-based vaccine.

To asses the effect of IL-6 transgene expression on a DC-based cancer vaccine, we have constructed a recombinant adenovirus, $AdV_{IL\text{-}6}$, expressing the mouse IL-6 gene. We then infected DCs with $AdV_{IL\text{-}6}$ and examined (i) the effect of $AdV_{IL\text{-}6}$ transfection on DC phenotype and function, and (ii) the antitumor effect of $DC_{OVA/AdVIL\text{-}6}$ vaccine in a BL6-10_{OVA} animal tumor model. As shown in our results, both the IL-6 transgene-engineered DC ($DC_{AdVIL\text{-}6}$) and the control $DC_{AdVpLpA}$ displayed higher expression of MHC class Ⅱ (I_a^b), CD40, CD54 and CD80 than untreated DCs, indicating AdV can enhance the maturation of DCs. Moreover, $DC_{OVA/AdVIL\text{-}6}$ vaccination stimulated stronger OVA-specific $CD8^+$ T cell and CTL responses compared to the control $DC_{OVA/AdVpLpA}$ vaccination. The tetramer analysis showed increased expression of OVA-specific $CD8^+$ T cells for $DC_{OVA/AdVIL\text{-}6}$ immunized mice. Also, activated T cells from $DC_{OVA/AdVIL\text{-}6}$ vaccinated mice showed significant OVA-specific killing of 93.3%, compared with only 46% killing activity derived from activated T cells from the control $DC_{OVA/AdVpLpA}$ vaccination mice. Animal studies showed that when challenged with a low dose (0.5×10^6) of BL6-10_{OVA} tumor cells, both vaccination with $DC_{OVA/AdVIL\text{-}6}$ and $DC_{OVA/AdVpLpA}$ induced complete protective immunity. Previous work has shown that peptide-pulsed

AdV-infected DC populations were sufficient to induce protection against a low dose challenge of tumor cells (67). Based on these results, we challenged with a higher dose (1×10^6) of BL6-10_{OVA} tumor cells to analyze the significant differences between $DC_{OVA/AdVIL\text{-}6}$ and $DC_{OVA/AdVpLpA}$ immunized mice. The mice vaccinated with $DC_{OVA/AdVpLpA}$ protected 50% (4/8) mice from challenge of high dose (1×10^6) of BL6-10_{OVA} tumor cells. On the contrary, vaccination with $DC_{OVA/AdVIL\text{-}6}$ still showed complete immune protection against the high dose challenge (8/8). This indicates that vaccination of mice with $DC_{OVA/AdVIL\text{-}6}$ is able to induce substantially more effective anti-tumor immunity *in vivo* than $DC_{OVA/AdVpLpA}$. The enhanced anti-tumor immunity of the $DC_{OVA/AdVIL\text{-}6}$ may be derived from transgene IL-6 expression.

To elucidate the immune mechanism underlying the result of $DC_{OVA/AdVIL\text{-}6}$ vaccine, we generated the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T (T_B) and assessed their phenotypic characterization, survival and therapeutic effect in mice bearing solid EG7 tumors. It has been demonstrated that CTL are able to lyse target cells using two molecular mechanisms, one perforin/granzyme-based, the other Fas/FasL based (207, 208). The perforin/granzyme pathway is Ca^{2+}-dependent and antigen specific. In this pathway, the "pore-forming" protein perforin required to be polymerized and the polymerized perforin can act as a tube to guide the granzyme and the outside water into the target cell to lyse these cells. Moreover, this pathway occurs only when the target cells display specific antigen bound to MHC class I (209, 210). Unlike the Perforin/granzyme pathway, the Fas/FasL pathway does not require the presence of Ca^{2+}, and it induces antigen-independent and MHC-unrestricted target cell lysis (211, 212). In

addition, the time that it takes to lyse the target cell via perforin/granzyme pathway is shorter than via Fas/FasL. In our study, results showed that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T (T_A) expressed higher levels of FasL and perforin and displayed stronger tumor cell killing activity in mice bearing solid EG7 tumors, compared with the control T_B cells, indicating these cytotoxicity of T_A cells is mediated by both Fas/FasL pathway and perforin secretion. Moreover, this result show that the $DC_{OVA/AdVIL\text{-}6}$ stimulate $CD8^+$ T cells into effector CTL that had the higher cytotoxic ability, and can explain why the activated T cells from $DC_{OVA/AdVIL\text{-}6}$ vaccinated mice showed more significant OVA-specific antitumor immunity than the activated T cells from the control $DC_{OVA/AdVpLpA}$ vaccinated mice.

The result of the phenotypic characterization of $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T (T_A) and $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T (T_B), we found that the expression level of CD62L was higher in the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A), indicating $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells have a tendency to become memory T (Tm) cells. Moreover, after re-challenging mice, which had been cured of their tumors via $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells (T_A), with EG7 tumor cells 8 weeks subsequent to the tumor regression, the results showed that there was no tumor growth or temporary growth which disappeared within 20 days of the tumor inoculation. This suggests that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells become T memory cells. Based on the high level of CD62L on the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells and the results of this animal study, it was demonstrated that the IL-6-transgene engineered DC vaccine may increase $CD8^+$ memory T cell development.

The *in vivo* persistence of DC-activated $CD8^+$ T cells is an important factor for a DC-based vaccine. Unfortunately, the vast majority of activated $CD8^+$ T cells die quickly due to AICD via apoptosis, which greatly limits treatment efficacy. In our study, we found that the number of detected OVA-specific $CD8^+$ T cells in peripheral blood of mice treated with $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells and the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells accounted for 2.54% and 2.37% of the total $CD8^+$ T-cell population at day 6 after adoptive transfer. However, the numbers gradually dropped to 1.45% and 1.00% at day 12, and 1.23 and 0.38% at day 30 after adoptive transfer, indicating that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells have prolonged survival *in vivo*, compared with the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells. It has been reported that the IL-6 leads to prolonged T-cell survival by preventing AICD, which is IL-2-independent and does not interfere the process of T-cell activation. (153). IL-6 mediated anti-apoptotic activity may account for the increased survival of the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells.

During clinical trails, patients usually bear existing tumor burdens. To correctly stimulate a clinical case, we built a mouse model bearing a 10-day established EG7 tumor for our animal study. These tumors were around 8 mm in diameter and had well-developed vasculature. Moreover, many human solid tumors can release immunosuppressive levels of IL-10 (99, 107). IL-10 has been identified as a key immunomodulatory cytokine that plays a central role in maintaining the proper balance between protective immunity against infections/tumors and limiting pro-inflammatory responses to self-antigens. It inhibits $CD4^+$ T cell proliferation and reduces

immunogenicity of DCs, leading to the inhibition of T-cell growth (105), T-cell anergy or the induction of Treg cells that suppress antigen-specific T cell responses (101, 106). Treg cells, as major immunosuppressive cells, play an important role in escape of tumor cells from immune control (107). It has been reported that addition of IL-6 to TGF-β can decrease the formation of Treg cells (116), suggesting IL-6 has the potential to counteract IL-10-induced immune suppression. The results of this animal study showed that 100% of mice treated with $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells were tumor-free, compared to the aggressive tumor growth seen in all 8 tumor-bearing mice treated with the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells, indicating that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells have enhanced antitumor activity. This may be the result of IL-6 counteracting Treg cell-mediated immune suppression.

Taken together, it has been demonstrated that $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells have prolonged survival and increased cytotoxic ability. These two characterizations may account for the immune mechanism underlying the result of IL-6 engineered-DC vaccine, including that $AdV_{IL\text{-}6}$ infected DCs can stimulate the proliferation of $CD8^+$ CTL and have strong antitumor immunity. IL-6 has the potential to convert Treg cell-mediated immune suppression. Moreover, the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells tend to become a memory T cells, indicating the IL-6 engineered-DC vaccine enhances memory T cell development. Based on these results, we suggest that IL-6 is a candidate for engineering DCs suitable for cancer vaccine development.

Chapter 7

CONCLUSIONS

In the immune system, DCs can take up, process and present TAAs to naïve T cells, and stimulate these naïve T cells to become CTLs. This is an important component in the initiation of the antitumor immune response. Moreover, IL-6 has proven to suppress AICD without interfering with the process of T-cell activation (153) and inhibit the Treg cell-mediated immune suppression (115, 116). In this thesis, we have utilized OVA-pulsed $AdV_{IL\text{-}6}$ infected DCs as a vaccine for cancer therapy. Upon DC maturation, we saw up-regulation of expression of MHC class Ⅱ (I_a^b), CD40, CD54 and CD80 in both $AdV_{IL\text{-}6}$-infected DCs and the control AdV_{pLpA}-infected DCs, indicating the AdV can enhance the maturation of DCs, independent of the transgene. OVA-pulsed $AdV_{IL\text{-}6}$-infected DCs ($DC_{OVA/AdVIL\text{-}6}$) stimulate a higher percentage of OVA-specific $CD8^+$ T cells and a stronger OVA-specific CTL response than the control OVA-pulsed AdV_{pLpA}-infected DCs ($DC_{OVA/AdVpLpA}$). Moreover, immunization with $DC_{OVA/AdVIL\text{-}6}$ induces more efficient protective immunity than the control $DC_{OVA/AdVpLpA}$ in C57BL/6 mice.

In the Part B of this study, we further elucidate the immune mechanism underlying the result of IL-6 engineered-DC vaccine by using the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells. Conclusively, the $DC_{OVA/AdVIL\text{-}6}$-engineered DC-activated $CD8^+$ T cells have a prolonged survival and stronger cytotoxic ability than the control $DC_{OVA/AdVpLpA}$-activated $CD8^+$ T cells. The $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells also have the tendency to become T memory cells. In the animal study, the $DC_{OVA/AdVIL\text{-}6}$-activated $CD8^+$ T cells have more

antitumor immunity in the EG7 solid tumor model than the control. Based on these results, AdV-mediated IL-6 transgene engineered DC vaccine can stimulate efficient $CD8^+$ T cell responses and antitumor immunity via prolonged T cell survival and enhanced T cell cytotoxicity.

Chapter 8

FUTURE DIRECTIONS

Although DC-based vaccines have showed durable tumor regression and potential cure of metastatic solid cancer in animal models, this immunotherapy has a very low tumor response rate in the range of 5%-10% of treated patients in clinic trail (170, 171). It has been found that the antitumor activity of activated tumor antigen-specific T cells is limited by the tumor cells escaping by releasing IL-10 and their short life in the body of patients. Immunosuppressive levels of IL-10 have been found in many human tumors (99, 107), and this IL-10 blocks tumor-specific helper T type 1 (Th1) responses (100), inhibits tumor-specific $CD8^+$ CTL cytotoxicity (108) and supports tumor growth (109, 110) by the induction of Treg cells ,which can suppress antigen-specific T-cell responses. Therefore, T-cell suppression derived from tumor-secreted IL-10 becomes one of the major barriers to immunotherapy for tumors. The short lifespan of the activated tumor antigen-specific T cells are another key problem in immunotherapy for cancer. Most of the DC-activated T cells in the vaccine died quickly via AICD, and only a small portion of effector CTLs survive to kill the target tumor cells, which limits the therapeutic effect of DC-based vaccines.

It has been suggested that IL-6 can overcome the IL-10-induced immune suppression by converting Treg cell suppression (115) and has anti-apoptosis activity for the active T cells (153). Moreover, in this study, we found AdV-mediated IL-6 transgene engineered DC vaccine stimulated $CD8^+$ T cell responses and antitumor immunity via prolonged T

cell survival and enhanced T cell cytotoxicity. Therefore, DCs engineered to secret IL-6 could perhaps be incorporated into a DC-based vaccine applicable to a clinical setting.

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